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WYETH PRODUCTS IN CLINICAL INVESTIGATION

Clinical Uses of WYDASE (Hyaluronidase):
One of the series of reports on current and future trends in therapy

In the June 1953, issue of "Surgery," in an extensive paper, "Clinical Uses of Hyaluronidase," Richard C. Britton, M.D., and David V. Habif, M.D., review the status of hyaluronidase in medical practice.

Hyaluronidase is valuable because of its ability to enhance the diffusion of fluids through tissues. It does this by temporarily altering the normal interstitial barrier, sometimes referred to as a cement substance, ground substance, or mesenchymal interfibrillar substance. Under clinical conditions hyaluronidase acts almost instantly to soften hyaluronic acid present in interstitial tissues. When it is used with fluid infusions or drug injections, diffusion and absorption are more rapid than without the enzyme.

Clinical Applications

Britton and Habif discuss the properties and current uses of hyaluronidase from the clinician's point of view, with special reference to the fields of surgery and surgical specialties, as follows:

General Surgery

- Hypodermoclysis
- Infiltration and block anesthesia
- Lymphatic marking
- Dispersion of postoperative edema

Minor Surgery

- Nerve block anesthesia
- Infiltration anesthesia
- Traumatic or postoperative hematomas

Plastic Surgery

- Local anesthesia
- Protection of flaps and grafts from edema and hematoma
- Treatment of keloids

Drug Therapy

- Injection of medications

Ophthalmology

- Bulb anesthesia
- Retrobulbar infiltration
- Temporofacial nerve block
- Local anesthesia for operative procedures

Urology

- Adjunct in control of lithiasis
- Excretory urography
- Hemostasis in transurethral resection
- Local anesthesia in adult circumcision
- Reduction of paraphimotic edema

Obstetrics and Gynecology

- Pudendal block anesthesia

Otorhinolaryngology

- Local anesthesia
- Treatment of sinusitis with local antibiotics

Orthopedics

- Acute ankle sprains
- Bursitis and tendinitis
- Reduction of simple fractures
- Dispersion of fluid collections in joints and bursae

Dental and Oral Surgery

- Mandibular block anesthesia
- Buccal and facial hematoma
- Reduction of trismus

In summary, Britton and Habif conclude: "Hyaluronidase is one of several enzymes recently developed to enhance and supplement therapeutic measures. Its value has been well established in speeding the administration and absorption of parenteral fluids and drugs, in producing greater area and effectiveness of local infiltration anesthesia, in treating specific metabolic disorders, and in dispersing and enhancing diffusion of traumatic and pathologic fluid collections."

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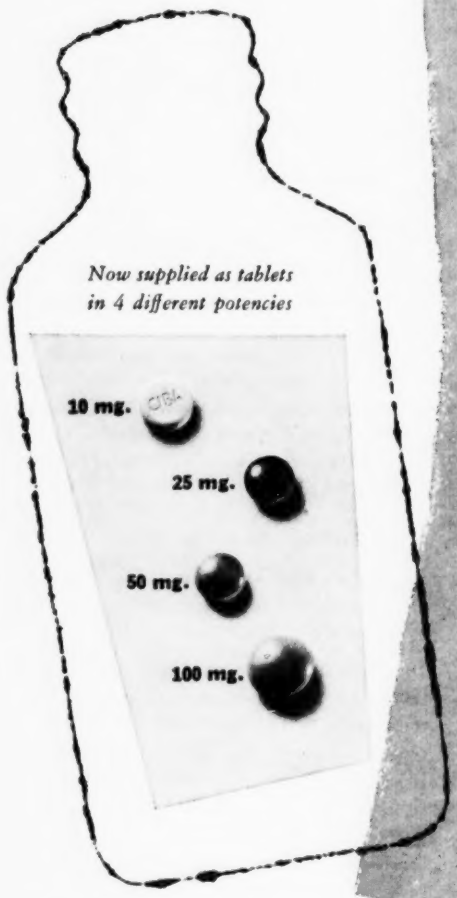

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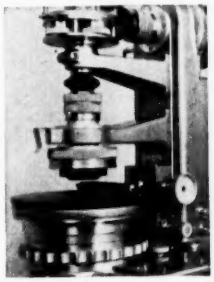
Natural and synthetic fibers, single crystals, tissue sections, bacterial cultures – all have been examined and have yielded excellent, reproducible infrared spectra by means of a new infrared microscope attachment for Perkin-Elmer Model 12, 112, and 13 Spectrometers. Useful spectra have been obtained with as little as 0.1 microgram of sample. The new microscope should find wide applications in crystal and fiber studies as well as in medical infrared studies.



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Infrared Analysis Opens New Avenues in Medical Research

The possibilities of infrared in the medical field can best be considered in three classifications, based essentially on the nature and origin of the sampling involved. The first, and at present the most fruitful, is the use of infrared in the analytical research and production of chemicals of direct interest to medicine and allied fields.

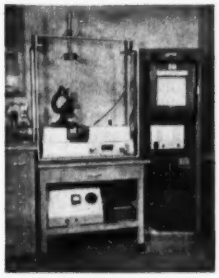
The second concerns the application of infrared to the study of naturally occurring biological samples which can be extracted and handled as chemical mixtures; and the third involves the study of material in situ, as tissues, sera, bacteria, etc.

• **Value of Infrared**—The primary value of infrared to the biochemist or pharmaceutical chemist lies in the uniqueness of spectral characteristics, small sampling, rapidity of analysis, and the recovery of unchanged sample.

• **Steroid Studies**—A classic example is the fundamental work of the late Dr. Konrad Dobriner on the role of steroids in metabolism. In this case, steroids extracted from urine presented an analytical problem which could be solved by infrared methods only.

• **Tissue Work**—Usually, tissue samples are prepared by microtoming a 10-50 micron frozen section, placing it on an infrared plate and drying it at low temperature. Schwarz, Mellors and Blout have shown various spectra, both normal and pathological.

• **Materials vs. Tissues**—It would seem that an attack on the determination of the chemical materials present in tissues must be made by the methods of Dobriner using selective extraction, fractionation into low component mixtures, with spectroscopy as the final analysis. The problem is formidable because of the large number of chemically and spectroscopically similar materials present.



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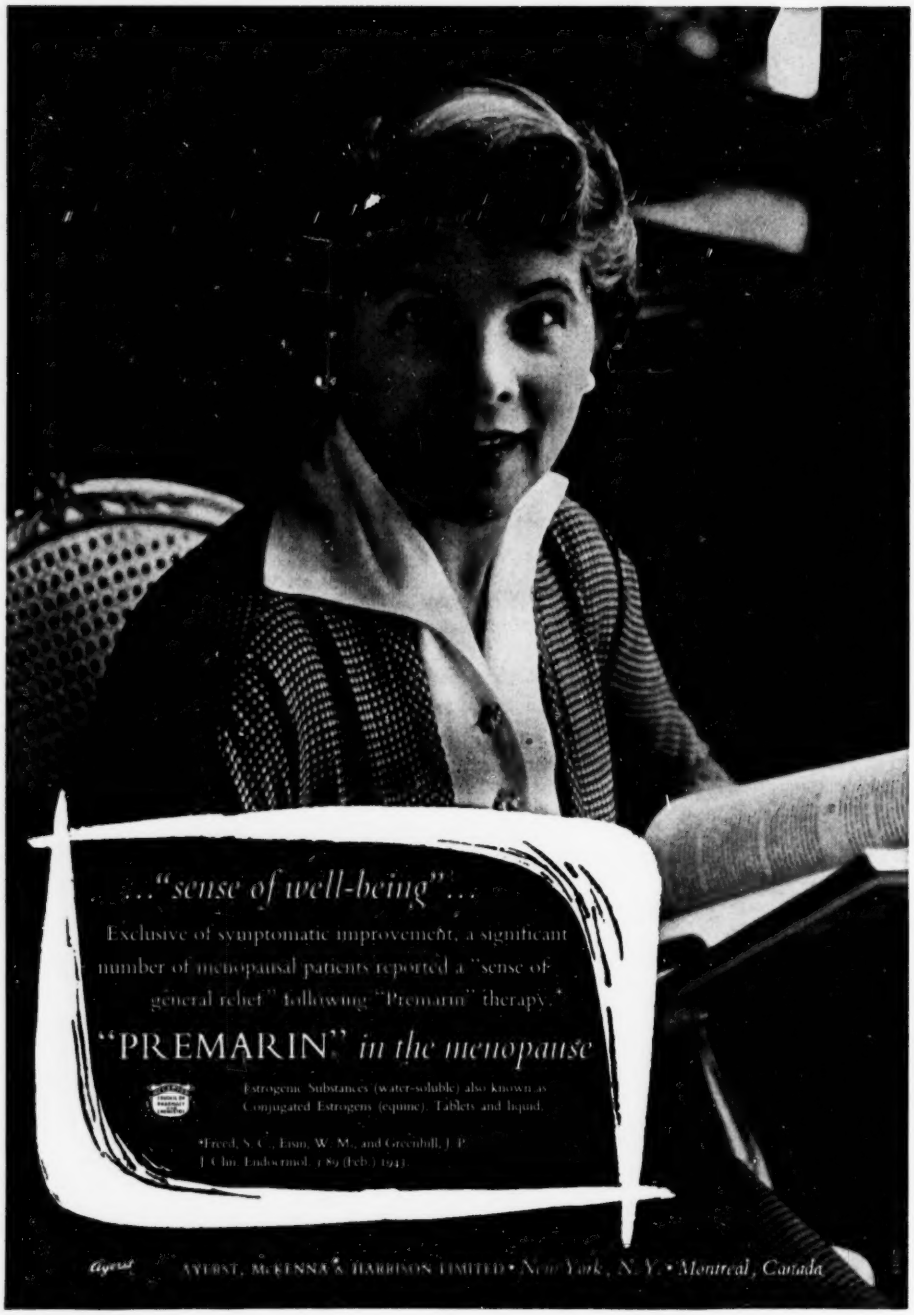
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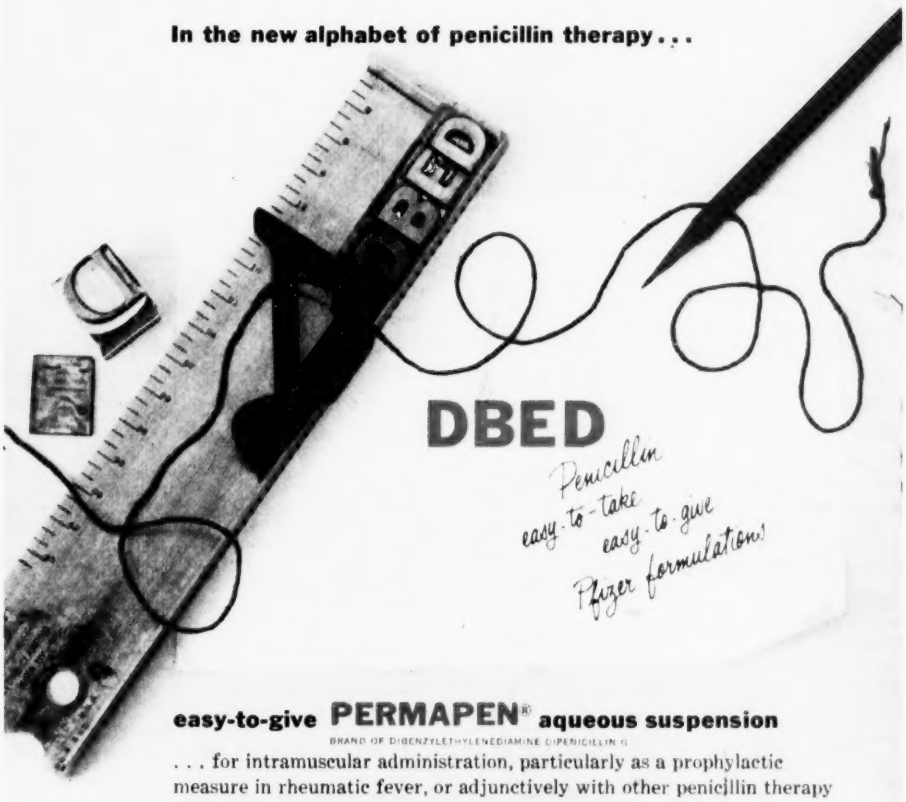

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PARTIAL PURIFICATION OF HUMAN PROTHROMBIN AND PROCONVERTIN: THEIR CHARACTERISTICS AND INTERACTION [1]

By JESSICA H. LEWIS AND JOHN H. FERGUSON

(From the Department of Physiology, University of North Carolina, Chapel Hill, N. C.)

(Submitted for publication April 6, 1953; accepted May 22, 1953)

Independently and almost simultaneously a number of investigators recognized the fact that at least two plasma factors are important in the conversion of prothrombin to thrombin in the presence of *tissue* thromboplastin and calcium. Various names have been applied to these factors and, at a recent Macy Conference on blood clotting (1), most of the investigators agreed that they could be grouped into two entities as follows:

Relatively labile factor	Relatively stable factor
Labile factor (Quick [2, 3])	SPCA precursor (Alexander et al. [1, 13–20])
Factor V, Proaccelerin (Owren [4, 5])	Co-factor V, Proconvertin (Owren [1, 21, 22])
Plasma Ac globulin (Ware, Guest, and Seegers [6, 7])	Factor VII (Koller, Loeliger, and Duckert [23])
Prothrombin accelerator (Fantl and Nance [8, 9])	Prothrombin accelerator (MacMillan [24])
Plasmatic co-factor of thromboplastin (Honorato, Rojas, and Ivanovic [10, 11])	Co-thromboplastin (Mann and Hurn [25, 26])
Plasma prothrombin conversion factor (Stefanini [12])	Stable prothrombin conversion factor (Owen, Bollman, and Magath [27, 28])

Without prejudice and for the sake of simplicity, we have chosen to use the terms proposed by Owren, namely, proaccelerin and proconvertin for the plasma factors, and accelerin and convertin for the active (serum) factors.

The investigations presented in this paper deal with a method for the partial separation of prothrombin and proconvertin from human plasma and the study of their interactions.

MATERIALS

Imidazole buffer: pH 7.3 (26).

Accelerin (Accelerator Globulin, AcG): Barium carbonate treated beef serum prepared according to the method of Ware and Seegers (29). For the purposes of the present study this crude material served as a satisfactory source of accelerin, free from prothrombin and proconvertin, and retained full potency on storage at −20° C. for one year or longer. In additional experiments, which will be reported separately, we have employed highly purified human proaccelerin. This material was not used in the present experiments as, due to its marked storage lability, it must be prepared specially for each individual experiment, and because we wished to study the reactions of proconvertin and prothrombin in the presence of accelerin rather than proaccelerin.

Thromboplastin: Soluplastin, obtained through the courtesy of Dr. E. W. Blanchard, Schieffelin and Company. Dog lung thromboplastin, freshly prepared fraction IV + VI of perfused dog lung, previously shown to have high thromboplastic potency as well as fibrinolysokinase activity (30).

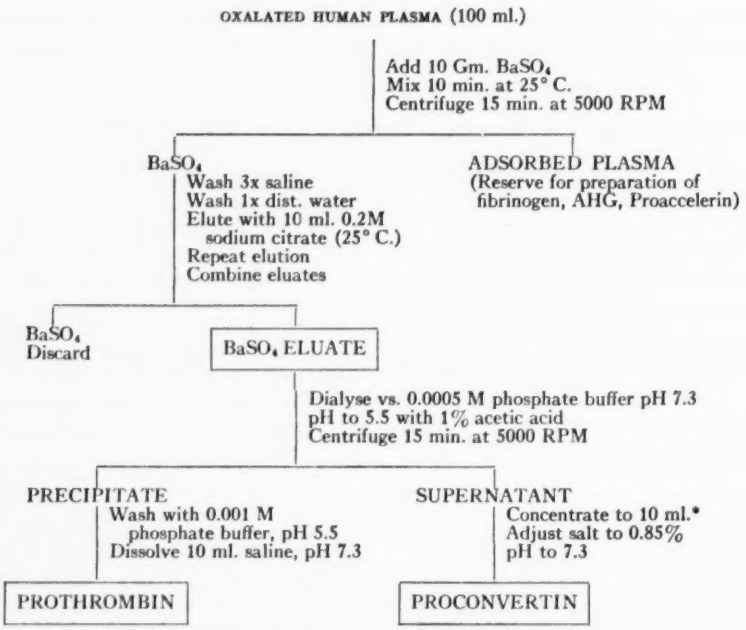
Fibrinogen: Human plasma fraction I (Cohn) prepared as a 0.7 per cent solution in 0.85 per cent sodium chloride (pH 7.3) and adsorbed with 50 mg. barium sulfate per ml. Although this material contains a high concentration of citrate, treatment with barium sulfate apparently did improve its stability. The fraction I was supplied through the courtesy of the American National Red Cross and Squibb and Company.

METHODS

Plasma fractionation: Many fractionation procedures were attempted in the separation of prothrombin and proconvertin. Although the method outlined below does not supply absolutely pure fractions, it does yield sufficient separation of prothrombin and proconvertin to allow study of the properties of these materials. All procedures were carried out at 4° C. unless otherwise stated. Various attempts to further purify the two fractions were made, but repeated reprecipitation and readsorption and elution from barium sulfate did not lead to preparations of significantly greater purity.

Thrombin activation mixtures: All activation mixtures (except controls in Table I) contained 0.5 ml. imidazole buffer, 0.5 ml. thromboplastin (diluted 1 to 5 with saline), 0.2 ml. accelerin (diluted 1 to 16 with saline) and 0.5 ml. 0.15 M $CaCl_2$. Prothrombin and proconvertin were added in 0.1 ml. amounts unless otherwise stated, and in all cases, sufficient 0.85 per cent sodium chloride was added to

[1] This investigation was supported in part by a research grant from the Division of Research Grants of the National Institutes of Health, U. S. Public Health Service.


OXALATED HUMAN PLASMA (100 ml.)
Add 10 Gm. BaSO4
Mix 10 min. at 25° C.
Centrifuge 15 min. at 5000 RPM
BaSO4
Wash 3x saline
Wash 1x dist. water
Elute with 10 ml. 0.2M sodium citrate (25° C.)
Repeat elution
Combine eluates
ADSORBED PLASMA
(Reserve for preparation of fibrinogen, AHG, Proaccelerin)
BaSO4 Discard
BaSO4 ELUATE
Dialyse vs. 0.0005 M phosphate buffer pH 7.3
pH to 5.5 with 1% acetic acid
Centrifuge 15 min. at 5000 RPM
PRECIPITATE
Wash with 0.001 M phosphate buffer, pH 5.5
Dissolve 10 ml. saline, pH 7.3
SUPERNATANT
Concentrate to 10 ml.*
Adjust salt to 0.85%
pH to 7.3
PROTHROMBIN
PROCONVERTIN

* Concentration may be accomplished by evaporation from a dialysis bag, evaporation in vacuo, dialysis against concentrated PVP, or lyophilization. The first method was usually employed.

bring the total volume to 5 ml. Employing the $BaSO_4$ Eluate (previously dialyzed to remove sodium citrate), which contains both prothrombin and proconvertin, the above concentrations of $CaCl_2$, thromboplastin, and accelerin were found to be optimal.

Activation and clotting were carried out at 25° C. Experiments showed that a greater yield of thrombin and more reproducible results were obtained when activation was carried out in siliconed tubes, which prevent the adsorption of the formed thrombin onto glass. At intervals, 0.2 ml. samples of the activation mixtures, in siliconed tubes, were removed with siliconed pipettes and added to 0.2 ml. fibrinogen, in glass tubes, and the clotting times obtained. The concentration of each lot of prothrombin was adjusted so that approximately a 10 second clotting time was obtained after complete activation under optimal conditions.

Thrombin "unitage": For simplification in interpretation of the results we have converted clotting times to "units." The most active clotting mixture, after full activation, was arbitrarily chosen as containing 100 thrombin "units" (per 0.2 ml.). Precise dilutions of this mixture were prepared in siliconed tubes, employing, as diluting fluid, the same materials as in the activation mixture but without the prothrombin or proconvertin. Clotting times for each dilution were obtained in triplicate, and a curve, relating concentration of the thrombin mixture and clotting time, constructed. By reference to this curve, clotting times could then be converted to these arbitrary "units."

The results presented below are those of individual experiments, performed in duplicate, each of which is illustrative of a number of similar experiments carried out on different lots of prothrombin and proconvertin.

TABLE I

*Controls (run 4)**

Mixtures	Soluplastin (*dil. 1–5*)	AcG (*dil. 1–16*)	Prothrombin	Proconvertin	Thrombin yield "*units*"	Incubation time (25° C.) *minutes*
A	0.5	0.2	0.1	0.3	100.0	3
B	0.5	0.2	0.1		95.0	10
C	0.5	0.2		0.3	4.0	3
D	0.5		0.1	0.3	5.5	60
E		0.2	0.1	0.3	1.0	60

* All activation mixtures contained 0.5 ml. 0.15 M $CaCl_2$, 0.5 ml. imidazole buffer, and the reagents listed above in a total volume of 5 ml.

RESULTS

Identification and purity of reagents: In order to establish the purity of our reagents a number of control tests were carried out.

Five ml. mixtures containing 0.5 ml. imidazole and the other ingredients (calcium, thromboplastin, accelerin, prothrombin, proconvertin) either singly or in any of the possible combinations were tested for thrombin formation at intervals during a one hour incubation period. Appreciable thrombin was formed only in those mixtures shown in Table I, which is illustrative of a typical run. The complete mixture (A) activated rapidly, and this mixture was designated as yielding 100 "units" of thrombin. In the absence of proconvertin (B), 95 "units" of thrombin were formed, but the time of complete activation was considerably slower. When proconvertin was activated in the absence of added prothrombin (C), 4.0 "units" of thrombin were formed. If accelerin or thromboplastin were omitted (D,E) there was only slight activation during a one hour period.

It seems probable that a pure prothrombin preparation would *not* activate in the absence of proconvertin. In one experiment, the possibility that proconvertin might be present as a contaminant of thromboplastin was explored. Various thromboplastin preparations (rabbit brain, dog brain, beef lung, dog lung, cephalin) were substituted for soluplastin, but in each case some activation of the prothrombin occurred.

All of our prothrombin preparations have activated in the absence of added proconvertin, yielding from 70 to 100 per cent of the expected quantity of thrombin. We have roughly estimated the quantity of contaminating proconvertin from the incubation time necessary to reach maximal activation. This has varied from 10 to 30 minutes with different lots of prothrombin. The quantity of prothrombin contaminating a proconvertin preparation can be readily assayed, as it is rapidly converted to thrombin. In different fractionation runs the proconvertin preparations have formed from 0.5 to 6 "units" of thrombin.

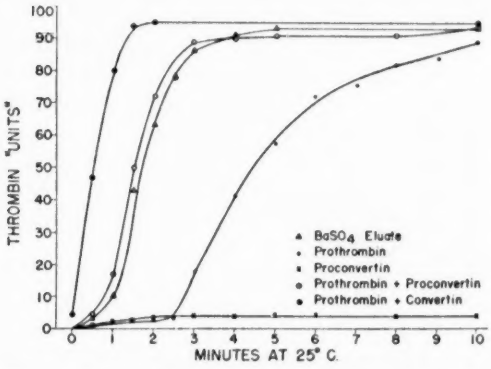


FIG. 1. THROMBIN FORMATION FROM HUMAN $BaSO_4$ ELUATE, PROTHROMBIN, AND PROCONVERTIN

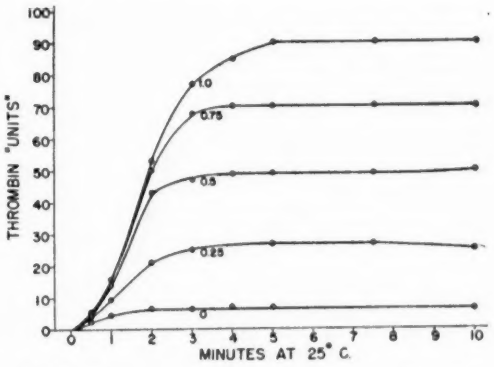


FIG. 2. EFFECT OF VARYING PROTHROMBIN CONCENTRATION

The prothrombin solution was diluted 1 to 10 and employed in 1.0, 0.75, 0.5 and 0.25 ml. amounts. All tests contained 0.2 ml. proconvertin.

In Figure 1, the results of activation of the $BaSO_4$ Eluate and its two fractions, "prothrombin" and "proconvertin," are compared. The $BaSO_4$ Eluate activated rapidly, while the prothrombin activated much more slowly, but yielded almost as much thrombin. The proconvertin also activated rapidly, but the thrombin yield was low. When the prothrombin and proconvertin fractions were mixed, the activation curve closely resembled that of the initial $BaSO_4$ Eluate. The most rapid activation was observed in a test in which convertin, formed by pre-incubation of the proconvertin with calcium and thromboplastin, was added to the prothrombin (see below).

Effects of varying prothrombin concentration: In this experiment (Figure 2) prothrombin in varying amounts was activated in the presence of a standard concentration of proconvertin. The thrombin yields, allowing for that formed from the proconvertin, were found to be proportional to the

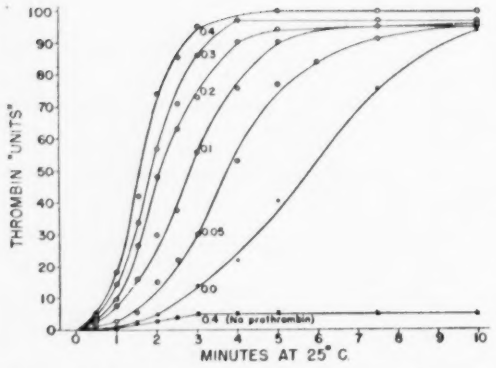


FIG. 3. EFFECTS OF VARYING PROCONVERTIN CONCENTRATION

added prothrombin concentrations. The proconvertin alone (curve 0) yielded 6 "units" of thrombin. If these 6 "units" were subtracted from the observed thrombin "units," they became proportional to the initial prothrombin concentrations: 84, 64, 44, 21 : : 1.0, 0.75, 0.5, 0.25.

The same concentrations of prothrombin were activated in the absence of added proconvertin (not illustrated). The activation rates were very slow, the maximal thrombin yield (between 70 to 80 per cent of that expected) being obtained after 15 to 30 minutes incubation.

Effects of varying proconvertin concentration: In this experiment the prothrombin concentration was maintained constant, and the proconvertin concentration varied. The results (Figure 3) clearly show that the rate of activation of prothrombin (in the presence of standard concentrations of calcium, tissue thromboplastin, and accelerin) is dependent upon the concentration of proconvertin. The small differences in final thrombin yields may be accounted for by the varying amounts of prothrombin present as a contaminant of the proconvertin.

Activation of proconvertin: In even the highest concentration of proconvertin employed in the experiment illustrated in Figure 3, there was an initial lag in thrombin formation during the first minute of incubation. Pre-incubation of the proconvertin with thromboplastin and calcium, as first suggested by Mann (25) in his studies on co-thromboplastin, eliminates this lag (see Figure 1). Experiments not illustrated showed that the addition of accelerin during the pre-incubation was not essential to this conversion of proconvertin to convertin. Pre-incubation of the proconvertin with either calcium or thromboplastin alone did not cause the formation of convertin. Additional experiments also showed that the addition of thrombin to the usual proconvertin-prothrombin mixture did not eliminate the initial lag in thrombin formation.

Rate of activation of proconvertin: In a series of identical mixtures, the proconvertin was incubated with calcium and thromboplastin for varying lengths of time before addition to the prothrombin and accelerin. The results are illustrated in Figure 4. After 10 minutes pre-incubation of proconvertin, the initial lag in thrombin formation had been eliminated. Longer pre-incubation did not increase the convertin activity.

Effects of varying convertin concentration: In this experiment, various amounts of proconvertin were pre-incubated for 10 minutes with calcium and thromboplastin before addition to prothrombin and accelerin. The results, illustrated in Figure 5, show that the rapidity of activation of prothrombin was dependent upon the convertin concentration.

Action of thromboplastin: The experiments presented above showed that calcium and thromboplastin convert proconvertin to convertin. We attempted to determine whether calcium and thromboplastin were also important in the conversion of prothrombin to thrombin in the presence of *convertin* and accelerin. In order to do this, proconvertin was incubated with calcium and thromboplastin and then attempts were made to remove these activators. Although a number of

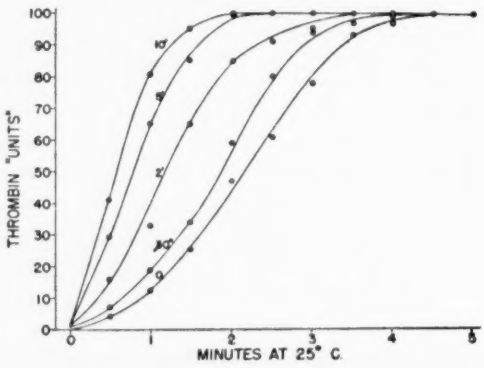


FIG. 4. ACTIVATION OF PROCONVERTIN

procedures for the removal of calcium (passage through the ion-exchange resin, Dowex 50, oxalation, citration, and dialysis) were tried, no convertin activity could be demonstrated after removal of the calcium.

On the other hand, substituting freshly prepared dog lung thromboplastin for soluplastin, it was possible to show full convertin activity after removal of most of the thromboplastin by high speed centrifugation. The results are shown in Figure 6. In the first test (A), calcium, dog lung thromboplastin, and proconvertin were incubated for 10 minutes and then added to prothrombin and accelerin. In the next test (B) proconvertin was again incubated with thromboplastin and calcium, and the mixture was centrifuged at 20,000 RPM for 20 minutes before adding to the prothrombin and accelerin. In test (C) the same procedure of incubation and centrifugation was followed as for test (B), but fresh thromboplastin was added along with the prothrombin and accelerin in the final activation mixture. In the last test (D), the calcium and thromboplastin were incubated, without proconvertin, and centrifuged before adding to the prothrombin, proconvertin, and accelerin. This test shows that a trace of thromboplastin remained after the centrifugation, as some thrombin was formed. In the absence of thromboplastin, no activation was observed. The similarity between curves (A) and (C) and their striking difference from curve (B) suggest that convertin is present after the removal of thromboplastin, but that it can fully activate the prothrombin only in the presence

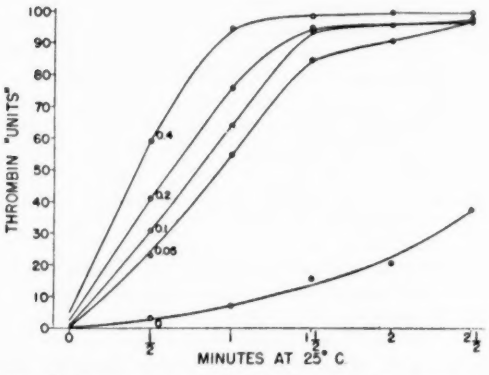


FIG. 5. EFFECTS OF VARYING CONVERTIN CONCENTRATION

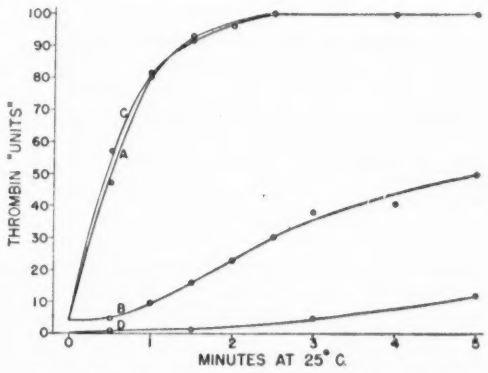


FIG. 6. ACTION OF THROMBOPLASTIN (SEE TEXT)

of adequate thromboplastin, as well as accelerin and calcium.

Stability of prothrombin and proconvertin: In order to test the stability of prothrombin and proconvertin, samples were heated to various temperatures, while control samples were kept at 4° C. In Table II the residual activity, after treatment, is recorded as percentage of that in the control samples. Proconvertin activity was estimated by comparison to a series of activation curves in which the concentration of the control proconvertin was varied. The results suggest that both fractions are relatively heat labile.

DISCUSSION

These investigations show that human prothrombin and proconvertin can be partially separated from oxalated blood plasma by a simple fractionation procedure. Koller, Loeliger, and Duckert (23) and Owren (1, 22) have accomplished a similar separation of these factors from bovine blood by using a differential adsorption method. However, they state that this technic does not work on human blood. The excellent studies of Alexander and his co-workers (1, 13–20) have shown that human serum contains, in addition to accelerin, another factor, which they have called SPCA. Apparently SPCA is very similar to proconvertin and may prove to be a mixture of proconvertin and convertin. Our studies confirm many of the findings of these workers.

Activation of our human plasma fractions in the presence of standard concentrations of calcium,

TABLE II

Stability of prothrombin and proconvertin

Temperature	Residual activity	
	Prothrombin	Proconvertin
	%	%
37° C. for 4 hours	82	100
56° C. for 5 mins.	30	34
65° C. for 3 mins.	14	28

thromboplastin, and accelerin gave final thrombin yields which were quantitatively dependent upon the initial concentration of prothrombin, but not of proconvertin. On the other hand, rate of thrombin formation was dependent upon proconvertin concentration and, in the presence of a fixed amount of prothrombin, the quantity of thrombin measured in early stages of activation was almost directly proportional to the proconvertin concentration. This strongly suggests that the ordinary one-stage prothrombin time (Quick test), in the presence of normal proaccelerin, largely reflects proconvertin, rather than prothrombin concentration.

Our findings that calcium and thromboplastin (but not accelerin) activate proconvertin to convertin extend the data of Mann and Hurn (25, 26), Landwehr and Alexander (18), and Owren (1), which previously pointed to some non-prothrombin plasma factor being activated by calcium and thromboplastin. In our cited isolated systems, complete activation of proconvertin by calcium and thromboplastin required about ten minutes, but other experiments, not illustrated, indicate that the rate of convertin formation is very dependent upon the potency of the thromboplastin. This observation might explain the fact that fractions, which we have prepared from spontaneously clotted serum, have contained primarily proconvertin.

During spontaneous plasma coagulation, thrombin formation is very slow during the first few minutes. This "lag phase" can be partially eliminated by introducing tissue thromboplastin, which by-passes the physiological action of a number of plasma substances and platelets. In our isolated system, we are able to reduce still further this "lag phase" by employing accelerin instead of proaccelerin. Complete elimination of the "lag phase" is accomplished by substituting convertin for proconvertin.

Additional experiments showed that convertin was unable to form thrombin from prothrombin unless calcium, thromboplastin, and accelerin were also present.

CONCLUSIONS

1. The final thrombin yield, in the presence of standard amounts of calcium, thromboplastin, accelerin, and proconvertin, is dependent upon the initial prothrombin concentration.

2. The rate of thrombin formation, in the presence of standard amounts of calcium, thromboplastin, accelerin, and prothrombin, is dependent upon the proconvertin concentration.

3. Proconvertin is activated to convertin by calcium and thromboplastin. The presence of accelerin is not necessary.

4. The "lag phase" in thrombin formation in a mixture containing prothrombin, thromboplastin, calcium, accelerin, and proconvertin can be eliminated by pre-incubation of the proconvertin, calcium, and thromboplastin.

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HEMODYNAMIC EFFECTS OF 1-HYDRAZINOPHTHALAZINE (APRESOLINE) IN HUMAN PREGNANCY: RESULTS OF INTRAVENOUS ADMINISTRATION [1]

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Gross, Druey and Meier (1) first demonstrated that 1-hydrazinophthalazine (Apresoline) [3] given intravenously or orally to experimental animals, produces a marked and prolonged hypotensive effect, with simultaneous increases in pulse rate, auricular pressure, and renal blood flow.

Further animal experiments showed that this drug is effective against both neurogenic and renal hypertension (2, 3, 4), and that its hemodynamic effects are different from those of typical autonomic or adrenergic blocking agents (1, 5). Marked inhibition and even reversal of the vasopressor effects of epinephrine by Apresoline have been observed by some investigators (1, 2, 6, 7), whereas others, despite the use of large doses of the latter drug, have obtained only slight if any inhibition of such effects (8, 9). Apresoline does not seem to alter the pressor response to hypoxia or to the carotid sinus reflex (6, 9). It has been suggested that it may act through the hypothalamus or that it may counteract the effects of circulating vasopressor substances (1, 3, 4, 10). The drug increases cardiac output and renal blood flow in unanesthetized animals, whereas renal blood flow remains unaltered in anesthetized animals (7).

Patients with hypertension of diverse etiology seem to respond to the vasodepressor action of Apresoline, even when hypertension has persisted or recurred after sympathectomy (3, 4, 10). The induced fall in arterial blood pressure is usually accompanied by an increase in renal plasma flow (11, 12) and a decrease in cerebral vascular resistance (13). In man, inhibition or abolition of vasopressor reflexes by Apresoline, as well as inconsistent effects on the vasopressor action of epinephrine have been reported (2, 14, 15).

In the present study, the hemodynamic effects of Apresoline were investigated in normotensive non-pregnant and pregnant women, in patients with toxemia of pregnancy and with essential hypertension associated with pregnancy. As has been previously reported (16, 17, 18), ganglionic blocking agents induce only a negligible fall in blood pressure in patients with acute toxemia of pregnancy. In contrast, in normal pregnant women and frequently in pregnant patients with essential hypertension these drugs have a marked hypotensive effect, usually associated with a decrease in both cardiac output and renal plasma flow (19, 20). It was considered possible that the variation in response to ganglionic blocking agents of these different groups of patients together with the information gathered from determinations of the responses to Apresoline in comparable groups of subjects might clarify the hemodynamic effects of this drug and perhaps the general problem of toxemia of pregnancy.

CLINICAL MATERIAL

The clinical material for this study consisted of 75 subjects grouped as follows: 12 healthy normotensive non-pregnant women; 12 normotensive pregnant subjects; 33 patients with toxemia of pregnancy (29 with pre-eclampsia and 4 with convulsive eclampsia); and 18 pregnant patients with essential hypertension. The diagnosis of toxemia of pregnancy and of essential hypertension associated with pregnancy was made according to the criteria outlined elsewhere (16). The age of the patients varied from 16 to 39 years and the length of gestation ranged from 22 to 40 weeks.

[1] This investigation was supported (in part) by research grants from the National Heart Institute of the National Institute of Health, Public Health Service, and from Ciba Pharmaceutical Products, Inc., Summit, New Jersey.

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[3] Also known by the code numbers Ba-5968 and C-5968 and furnished through the courtesy of Ciba Pharmaceutical Products, Summit, N. J.

METHOD

With the exception of the four patients with convulsive eclampsia, who were studied upon admission, all the patients were at bed rest on the obstetric wards for at least 24 hours prior to the study. During this period, the spontaneous variations in blood pressure in the toxemic patients and those with essential hypertension were determined by sphygmomanometric readings every one or two hours, the pulse rate also being determined.

On the morning of the study, the patient was moved to a separate room and blood pressure readings, with the patient supine, were taken every one to two minutes for 10 to 50 minutes. The average of all these readings served as the control blood pressure. Apresoline was then given by rapid (20 seconds) single intravenous injection in doses ranging from 20 to 40 mg. Further blood pressure readings were taken every minute for the first half hour and then every five minutes for the next hour, subsequent readings being made at intervals of one-half to one hour until the blood pressure returned to control levels. The three lowest blood pressure readings within the first two hours after the injection were averaged to determine the maximal hypotensive response to the drug. The pulse rate was obtained from the radial or carotid artery. When more than one intravenous dose was given to the same patient, an interval of one to three hours was allowed between subsequent injections. During this time, the blood pressure and pulse rate had usually returned to control levels.

Venous cardiac catheterization was performed on three patients with toxemia of pregnancy according to the method of Cournand and Ranges (21). Cardiac outputs were determined on these patients by direct Fick principle, and pressures were recorded by means of a five channel optical oscillograph (Hathaway). Pulmonary "capillary" pressures were measured according to the method of Hellems, Haynes, and Dexter (22) and pulmonary arteriolar resistance calculated by the formula described by Fowler, Westcott, and Scott (23). Cardiac output determinations were also made on a series of patients with a high frequency ballistocardiograph (24). Although the validity of this method has been questioned, it is still believed by some authors (25) that relative values may be obtained from studies made on the same patient, particularly when the ballistic form is not abnormal. Mean arterial pressure was obtained either directly from the brachial artery or by adding one-third of the pulse pressure to the diastolic pressure. Blood volume studies (T-1824) were carried out according to Chinard's (26) modification of the single injection technique of Gregersen. Skin temperature was recorded in a constant temperature room with a Rauh electric pyrometer. Vasopressor reaction to cold and to the Valsalva

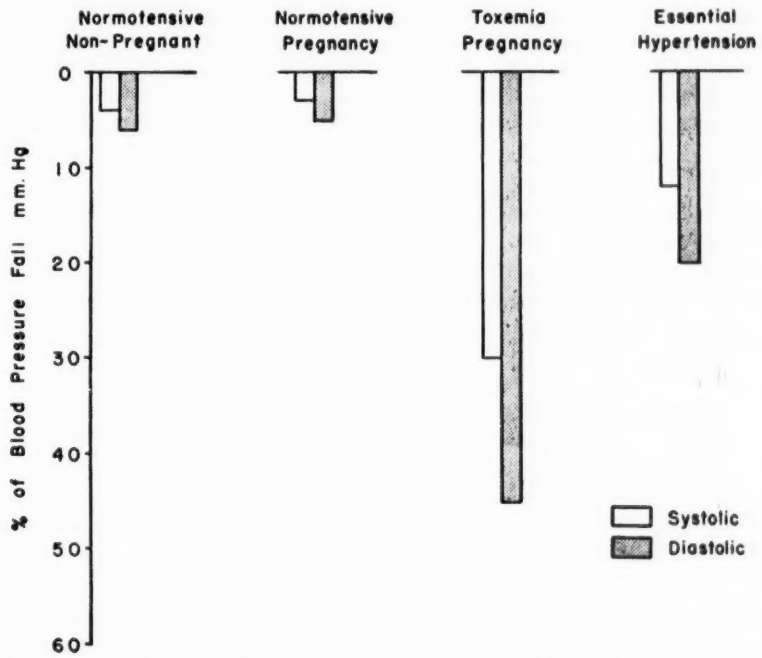

FIG. 1. BLOOD PRESSURE RESPONSES IN 75 PATIENTS WHO WERE GIVEN 163 INJECTIONS OF APRESOLINE

The figures represent per cent of change from the control blood pressure.

maneuver were determined once before and several times during the response to the drug. For the study of the Valsalva "overshoot," the blood pressure was recorded directly from the brachial artery. The effects of the drug on the vasopressor action of epinephrine were studied by giving single intravenous injections of varying doses of commercial epinephrine during the control period and again during maximal hypotensive response to Apresoline. After epinephrine injection, the blood pressure was recorded every one half minute; the pulse rate was obtained from electrocardiographic recordings.

RESULTS

1) *Blood pressure and pulse rate:* Blood pressure and pulse rate were determined in the 75 individuals given a total of 163 single injections. Three normotensive pregnant subjects and three toxemic patients were given 20 mg. doses. The remaining patients received doses of either 30 or 40 mg.

(a) *Normotensive nonpregnant and normotensive pregnant subjects:* Both groups of subjects responded in a similar manner. The average diastolic fall was 5 and 6 per cent, respectively; the change in systolic pressure was slight (Figure 1). Within this narrow range, the blood pressure response varied among the individual subjects even though the dosage was usually the same. In some patients the hypotensive effect lasted only for 30 minutes, whereas in others it persisted for approximately 2 hours. The pulse rate increased by 20 to 30 beats per minute without relationship to blood pressure response.

(b) *Toxemia of pregnancy:* The response of the patients with toxemia of pregnancy differed from that of normotensive subjects. Four to 15 minutes after the injection, the blood pressure began to fall gradually, the diastolic pressure before and more markedly than the systolic, and within 10 to 25 minutes after the injection, normal pressure levels were achieved and were maintained for several hours. The average fall in blood pressure was 30 per cent for systolic and 45 per cent for diastolic pressure (Figure 1). The average duration of the hypotensive effect of a single dose was 5 hours, although in three toxemic patients who received 20 mg., 30 mg., and 30 mg., respectively, the blood pressure remained low for 22, 24 and 25 hours, respectively, and then began to return gradually to control levels. The pulse rate invariably increased by 20 to 40 beats per minute after the injection but without relationship to the magnitude of fall in blood pressure.

(c) *Essential hypertension associated with pregnancy:* In this group, the average blood pressure fall was 11 per cent for systolic and 19 per cent for diastolic pressure (Figure 1). Here, again, the blood pressure did not begin to fall

TABLE I

Effect of Apresoline on cardio-pulmonary dynamics

Patient		(1) B.A.	(2) Mean B.A.	(3) C.O.	(4) C.I.	(5) P.C.	(6) P.A.	(7) Mean P.A.	(8) P.A.R.	(9) R.V.	(10) Mean R.A.	(11) R.V. Work	(12) T.P.R.
R. M. F.	Rest	178/102	130	7.82	4.77	8	32/15	20	123			0.75	1324
	After Apres.	162/78	105	12.67	7.73	13	33/15	23	63	33/2	9	1.55	664
G. T.	Rest	164/100	128	9.25	6.42	8	30/17	24	138			1.84	1102
	After Apres.	102/48	62	10.56	7.33	9	30/17	23	87	32/5	4	1.79	468
M. L.	Rest	203/127	158	4.47	2.81								2815
	After Apres.	125/52	80	7.63	4.80						4		834

(1) B.A.—brachial artery pressure mm. Hg.
(2) Mean B.A.—mean brachial artery pressure—mm. Hg.
(3) C.O.—cardiac output—litres per minute.
(4) C.I.—cardiac index—litres per minute per square meter.
(5) P.C.—mean pulmonary "capillary" pressure mm. Hg.
(6) P.A.—pulmonary artery pressure mm. Hg.
(7) Mean P.A.—mean pulmonary artery pressure mm. Hg.
(8) P.A.R.—pulmonary arteriolar resistance dynes. sec. cms^{-5}.
(9) R.V.—right ventricular pressure mm. Hg.
(10) Mean R.A.—mean right auricular pressure mm. Hg.
(11) R.V. work—mean right ventricular work kilogram meters per minute per square meter.
(12) T.P.R.—total peripheral resistance—dynes. sec. cm^{-5}.

TABLE II

The effect of Apresoline on skin temperature

Patient	Diagnosis	Skin temperature (° F.) before Apresoline			Skin temperature (° F.) after Apresoline*		
		B.P. *mm. Hg*	Toes	Fingers	B.P. *mm. Hg*	Toes	Fingers
F. D.	Toxemia	158/106	82	88	139/79	83	92
H. O.	Toxemia	140/98	84	90	119/69	84	96
B. C.	Toxemia	150/90	81	89	118/73	82	94
V. S.	Toxemia	153/101	83	87	140/87	83	97
B. M.	Toxemia	174/115	84	88	130/82	86	96
R. K.	Toxemia	180/110	82	89	140/80	84	95
J. T.	Ess. hypert.	204/106	82	88	170/92	83	92
R. B.	Ess. hypert.	184/110	82	86	140/98	83	95
O. W.	Ess. hypert.	160/105	82	87	130/90	82	93
S. D.	Normotensive	123/83	85	89	127/71	85	92
B. B.	Normotensive	120/84	84	90	124/68	86	93
N. G.	Normotensive	110/70	84	88	105/60	84	92

* The figures on skin temperature represent the average readings before and during the maximal Apresoline response.

until 5 to 20 minutes after the injection, and the maximal fall occurred 20 to 30 minutes after the administration of the drug. In all patients tachycardia of the same magnitude as in the preceding group was observed.

2) *Cardiac output, peripheral and pulmonary resistance:* The effects of Apresoline on the cardiopulmonary dynamics of three patients with toxemia are listed in Table I. Although the "resting" cardiac output and index were elevated in two patients (R. M. F. and G. T.), there was a further increase after injection of the drug. In the patient in whom the cardiac output was normal at rest, it was markedly increased after Apresoline. The pulmonary artery and pulmonary "capillary" pressures were normal at rest and did not vary consistently after the injection of the drug. The fall of the pulmonary arteriolar resistance was comparable to the marked fall of the total peripheral resistance.

Four patients with toxemia, four with essential hypertension associated with pregnancy, and three normotensive pregnant subjects were studied with the ballistocardiograph. None of the patients had abnormal ballistic forms either before or after the administration of the drug. The average control "cardiac output" in the toxemic and hypertensive group was 5.2 L. per min. increasing to 6.8 L. per min. after Apresoline; the total peripheral resistance was reduced 40 per cent. In the normotensive group, there was an average increase in the cardiac output of 600 cc. per min. and an average reduction of 18 per cent in the total peripheral resistance. The patients who were studied with the ballistocardiograph showed less individual variations than those studied with the Fick method in the values for cardiac output both before and after Apresoline.

3) *Skin temperature:* Skin temperature measurements were made in six patients with toxemia of pregnancy, three patients with essential hypertension associated with pregnancy, and three normotensive pregnant subjects. Before measurements were made, the patient was kept supine uncovered at a temperature close to 78° F. Readings were then taken from the fingers, hand, face, chest and abdominal walls, feet and toes.

The intravenous injection of Apresoline was followed by a significant rise in the temperature of the upper extremities, little change being observed in the temperature of the lower extremities (Table II and Figure 2). Significant rises also occurred in the temperature of the face and anterior wall of the chest. The changes in skin temperature showed no obvious relationship to the changes in blood pressure.

4) *Blood volume:* "Blood volume" changes were studied in four patients with toxemia of pregnancy and two normotensive pregnant subjects. Samples of venous blood for the dye determinations and hematocrit were obtained without stasis during the control periods and at the time of maximal hypotensive response to Apresoline. No significant changes in the blood volume were observed either in normotensive pregnant subjects or patients with toxemia of pregnancy.

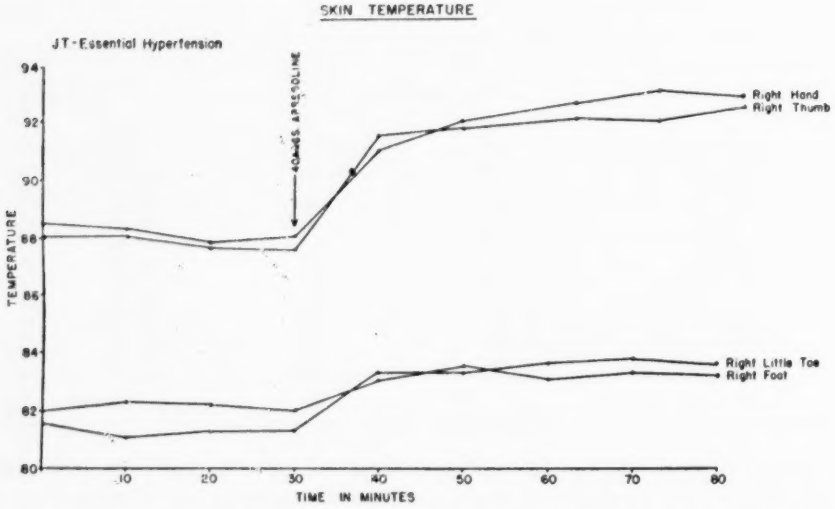

FIG. 2. PATTERN OF SKIN TEMPERATURE CHANGES AFTER INTRAVENOUS APRESOLINE

5) *Vasopressor reflexes:* Cold pressor tests were performed on five patients with toxemia of pregnancy, three pregnant patients with essential hypertension and two normotensive pregnant subjects by immersing the hand up to the wrist for one minute in ice water. Blood pressure readings were then taken every half minute. During the control period, a vasopressor reaction of varying magnitude was observed in all individuals tested. Following the injection of Apresoline, the pressor response to cold was unchanged (Table III). In five patients, the pressor response to the Valsalva maneuver was determined before and after the injection of the drug with direct blood pressure measurement. No significant changes in the Valsalva "overshoot" were observed.

6) *Vasopressor action of epinephrine:* The effect of Apresoline on the vasopressor action of epinephrine was studied in four normotensive pregnant subjects and four patients with toxemia of pregnancy. Epinephrine was given by single rapid intravenous injections in doses varying from 0.05 to 0.25 mg. before, and 20 to 30 minutes following, the administration of the drug (Table IV). The highest blood pressure reading within the first three minutes after the injection of epi-

TABLE III

Name	Diagnosis	Before Apresoline		After Apresoline	
		B.P. mm. Hg.	Response to Cold*	B.P. mm. Hg.	Response to Cold*
C.H.	Toxemia	130/100	19/8	120/70	20/7
A.C.	"	160/100	22/4	132/80	20/4
O.O.	"	200/120	8/3	140/90	9/4
V.S.	"	150/110	16/6	110/68	14/6
B.S.	"	130/92	10/4	105/70	11/3
I.F.	Ess. hypert.	160/100	21/5	150/98	19/5
H.O.	"	170/98	16/7	162/86	14/4
F.D.	"	150/102	17/6	140/92	16/6
S.O.	Normotensive	120/80	10/2	114/76	8/2
B.B.	"	122/78	6/3	118/74	7/2

*The response to cold was taken from the highest blood pressure reading recorded during the one minute test and the figures represent percent of rise from the control blood pressure and from the blood pressure level after Apresoline injection.

TABLE IV.

Effects of Apresoline on Vasopressor Action of Epinephrine.

Name	Diagnosis	Epinephrine Dose mg.	Before Apresoline				After Apresoline		
			B.P. mm. Hg.	Pulse	% of Change B.P.	% of Change Pulse	B.P. mm. Hg.	% of Change B.P.	% of Change Pulse
O.O	Toxemia	0.25	136/86	98	+105/63	+14	104/54	+140/141	+20
M.B.	"	0.25	140/97	88	+100/50	+ 9	115/79	+143/65	+21
M.S.	Normotensive	0.10	130/85	65	+ 92/41	+23	112/59	+123/116	+11
M.M.	"	0.10	110/66	76	+ 91/67	+10	107/60	+ 90/62	+15
R.S.	Toxemia	0.05	135/90	103	+ 56/27	0	129/76	+ 40/18	+ 2
E.J.	"	0.05	130/80	99	+ 55/25	+ 1	128/68	+ 48/18	+15
I.L.H.	Normotensive	0.05	101/67	81	+108/49	+19	80/48	+132/88	+10
F.A.	"	0.05	119/79	77	+ 85/47	+ 3	116/75	+ 55/33	+ 3

The percent of changes in B.P. and pulse rate before Apresoline represents the percent of the control blood pressure; that after Apresoline represents the percent of blood pressure level after the administration of the drug.

nephrine was taken as the maximal pressor response. Before Apresoline, the intravenous injection of epinephrine evoked a rise varying from 55 to 108 per cent in systolic pressure and from 25 to 67 per cent in diastolic pressure. After Apresoline, the epinephrine response was increased in five cases and slightly decreased in three. A decrease occurred in two individuals receiving doses of 0.05 cc. of epinephrine, although in one case, receiving this dosage, the epinephrine response was increased (Table IV). In no instance was the epinephrine response significantly inhibited or reversed.

The pulse rate showed variable changes after the first epinephrine injection, increasing in some subjects and decreasing in others. The tachycardia usually following administration of Apresoline was not significantly changed by the second epinephrine injection. Figure 3 illustrates epinephrine responses in a toxemic subject who received 0.25 mg. doses of epinephrine.

Tolerance: Tolerance to the drug was studied in 20 patients with toxemia who were given repeated intravenous injections of the same dosage of Apresoline. The blood pressure was allowed to return to control levels before a successive dose was administered. Table V shows the degree of fall in both systolic and diastolic blood pressure after successive injections. One fifth of the patients developed some tolerance after the second injection. However, with succeeding doses, the degree of tolerance became more marked as evidenced by the progressive increase in the number of patients who showed diminished response to the drug.

Side effects: Palpitation and a sensation of warmth were the most frequent complaints. Headache, "throbbing of the head," and dizziness were complained of by approximately 50 per cent of the entire group. Nausea and vomiting occurred in nine cases (two normotensive subjects, three patients with toxemia of pregnancy, and four pregnant patients with essential hypertension). These side effects were not related to the magnitude of response to the drug. In a few instances,

TABLE V

*Tolerance to Apresoline in toxemic patients**

Dose	No. patients	Patients with tolerance	Per cent fall systolic	Per cent fall diastolic
1st	20	0	30	42
2nd	20	4	20	36
3rd	20	11	17	25
4th	20	13	12	22
5th	15	11	10	13

* The blood pressure response is given as per cent of the average control blood pressure obtained before the administration of each dose. The degree of blood pressure fall listed after the first injection refers to those patients who developed tolerance.

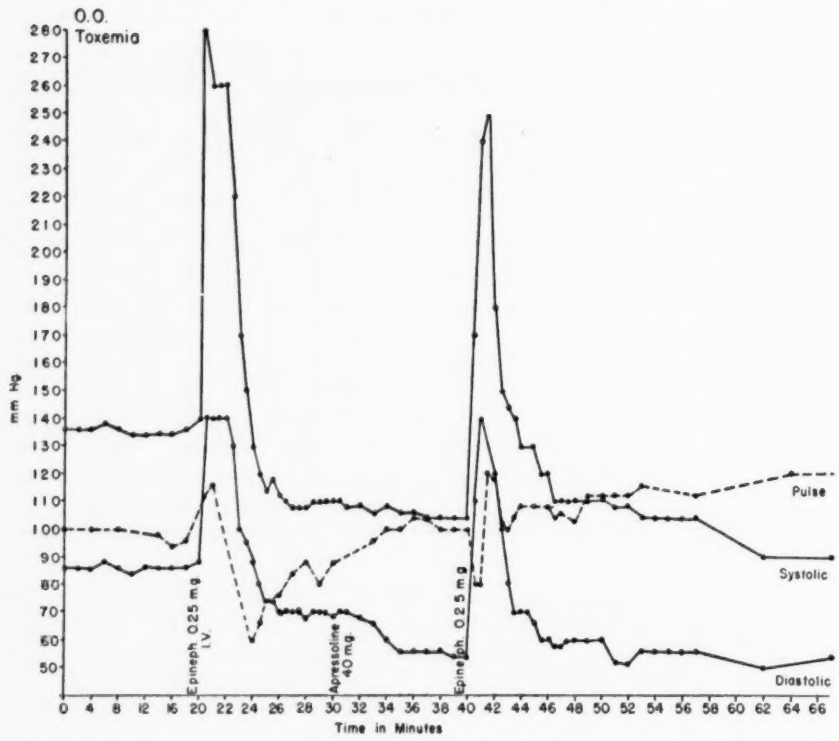

FIG. 3. PATTERN OF RESPONSE TO INTRAVENOUS EPINEPHRINE BEFORE AND AFTER APRESOLINE

anxiety, restlessness and epigastric pain were additional complaints.

DISCUSSION

Adequate explanation of the mechanism of action of Apresoline is not as yet available. The original observations of Gross, Druey, and Meier (1) showed that in animals the drug produces a significant decrease in the pressor effect of splanchnic stimulation and a partial to complete inhibition of the vasopressor action of epinephrine. These observations were supplemented by those of Freis and Finnerty (14) and Grimson, Chittum, and Metcalf (2) who observed some inhibition of vasopressor reflexes and concluded that the drug has certain "sympatholytic" properties. On the other hand, Britton, Taylor, and Ahlquist (6) and Walker and his co-workers (9), observed no significant action of Apresoline on the carotid sinus reflex or the pressor effect of hypoxia. Similarly, Craver, Barrett, Cameron, and Yonkman (5) found no evidence of sympatholytic activity of the drug in the cat.

The present data obtained from studies on pregnant subjects show that Apresoline failed to block the vasopressor reaction to cold and to Valsalva maneuver, even though the dosage employed was similar or higher than that used by Freis and his associates. The discrepancy between our results and those reported by these authors is not apparent.

Evidence has been presented (27, 28) that the vasopressor reactions to cold and to the Valsalva maneuver is mediated through the autonomic nervous system, and that this reaction can be inhibited by ganglionic blocking agents (17, 27, 28). The fact that Apresoline did not inhibit this reaction in pregnant women indicates that probably it does not act as a ganglionic blocker. This fact is further substantiated by Schroeder's observations that the drug remains effective after extensive sympathectomy. Also, the marked hypotensive effects

and increase in the cardiac output produced by Apresoline in toxemic patients in contrast to the slight effects observed in normal pregnancy, make this drug altogether different from the known ganglionic and adrenergic blocking agents. These observations, however, do not preclude the possibility that Apresoline might act through the autonomic nervous system by some as yet unknown mechanism.

Reports of the effects of Apresoline on the vasopressor action of epinephrine are inconsistent. Freis and his co-workers (14, 15) found no constant changes in the pressor response to epinephrine, whereas Walker and his co-workers (9) noted some depression after large doses of the drug. Moyer, Handley, and Huggins (7), on the other hand, have obtained in dogs complete abolition and even reversal of epinephrine pressor effects by Apresoline. Our observations are in agreement with those of Freis and would seem to indicate that Apresoline, in the dosage used, either has no effect on the vasopressor action of epinephrine, or may actually enhance its action. In fact, in the majority of instances, the vasopressor reaction to epinephrine and the associated subjective complaints were more marked after the administration of Apresoline than before.

Our results on the effects of Apresoline on the cardiac output, pulmonary dynamics and total peripheral resistance are in agreement with those observed by Wilkinson, Heikki, and Hecht (29). The fact that Apresoline increases markedly the cardiac output and at the same time reduces significantly the blood pressure and peripheral resistance suggests that its action may be limited to dilatation of the arteriolar system probably in the splanchnic bed and that the venous side of the circulation is not significantly affected.

Of interest is the rise in the skin temperature of the upper extremities as contrasted with the slight changes in that of the lower extremities. These findings differ from those seen after ganglionic blockade, which produces a more pronounced rise in the skin temperature of the lower extremities (30). These dissimilarities point to differences in mechanisms controlling blood flow to various segments of the body.

Several investigators (3, 10) have suggested that Apresoline may counteract the effects of circulating humoral agents. The fact that the drug is effective in reducing the hypertension of toxemia of pregnancy and that associated with acute nephritis (4, 31) lend support to this hypothesis, since available evidence suggests that the hypertension in these two disorders is not maintained by neurogenic mechanisms (16, 17, 32).

The delay in onset of the fall in blood pressure after intravenous administration of Apresoline has been observed by all investigators. It has been attributed by some (14) to a conversion of the drug to an active principle, but this possibility needs further investigation.

SUMMARY AND CONCLUSIONS

1) Hemodynamic effects of intravenous injection of Apresoline were studied in 12 normotensive non-pregnant, 12 normotensive pregnant, 33 toxemic and 18 pregnant subjects with essential hypertension.

2) Marked and prolonged blood pressure fall was observed in the toxemic group in contrast to a slight fall in the normotensive groups. Moderate blood pressure fall occurred in the essential hypertensive group.

3) Significant increase in cardiac output and reduction in the total peripheral and pulmonary arteriolar resistances occurred at the height of hypotension without any change in the blood volume. The temperature of the upper extremities rose more markedly than that of the lower extremities.

4) The drug failed to block the vasopressor reaction to cold and to the Valsalva maneuver in pregnant subjects.

5) The hemodynamic properties of Apresoline indicate that it may serve as a valuable adjunct in the treatment of toxemia of pregnancy.

ACKNOWLEDGMENT

The authors are indebted to Drs. John Braunstein, Ralph Scott and R. Helm and to Miss Mary Jane Tompkins for their assistance.

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STUDIES IN EDEMA. IV. WATER RETENTION AND THE ANTI-DIURETIC HORMONE IN HEPATIC AND CARDIAC DISEASE [1, 2]

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INTRODUCTION

Current opinion concerning the impaired output of water in patients suffering from cirrhosis of the liver (1, 2) or from congestive heart failure (3) emphasizes primarily the impaired excretion of sodium with consequent retention of water, and only secondarily tends to implicate the neurohypophysial antidiuretic hormone as a responsible agent (4, 5). The present studies and discussion are meant to explore more fully the physiology of this hormone in these diseases.

The relationship of the antidiuretic hormone to the abnormal retention of water in edematous states has been studied in several ways. Thus, following oral hydration with water, impaired water diuresis [5] has been demonstrated in patients with cirrhosis of the liver (4, 6, 7), and a water tolerance test based on this phenomenon has been employed for some years. A subnormal urinary excretion of water following intravenous hydration with 5 per cent glucose in water in some patients with cirrhosis has also been reported (4). Similarly, delayed water diuresis following the oral ingestion of water by patients suffering from congestive heart failure has been reported (8).

In order to establish the possible retention of water independent of that of sodium in hepatic and cardiac disease, investigators have sought for, and reported, increased amounts of an antidiuretic substance (ADS) in the blood (9) and urine of patients with cirrhosis of the liver (10, 11, 12) and in the urine of those suffering from congestive heart failure (13). Such increases in circulating and urinary ADS might be explained on the basis of overproduction of antidiuretic hormone, decreased "inactivation," [6] or a combination of both processes.

Small doses of Pitressin have been administered intravenously to patients with hepatic cirrhosis in order to study the rate of inactivation, which proved to be normal in these subjects (14, 15). To the extent that the inactivating mechanisms for endogenous antidiuretic hormone and Pitressin are similar, a decreased rate of inactivation of antidiuretic hormone seems to be excluded. Evidence suggesting that the inactivating mechanisms for endogenous antidiuretic hormone and Pitressin are indeed similar in patients with hepatic cirrhosis has been recently provided by Bernstein and co-workers (16). These investigators reported essentially similar antidiuretic responses of cirrhotic patients and normal subjects to Pitressin and to intravenous nicotine salicylate, which discharged endogenous ADH. Overproduction of this hormone, therefore, appears to be a more likely explanation for the reported increases of ADS in the blood and urine in cirrhosis (14), if ADS is indeed derived from or represents the neurohypophysial antidiuretic hormone. This will be discussed later in more detail.

[1] Presented in abstract form in the program of the Forty-Fourth Annual Meeting of the American Society for Clinical Investigation at Atlantic City, New Jersey on May 5, 1952.

[2] This study has been supported by a grant from the American Heart Association.

[3] Part of this work was done during the tenure of a Research Fellowship of the American Heart Association.

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[5] Water diuresis may be characterized with respect to the (a) time required to attain peak diuresis; (b) level of peak diuresis reached; and (c) proportion of an administered water load excreted during a test period. By definition, then, the attainment of peak diuresis may be "delayed," the level of this peak may be "reduced," and if the volume of water excreted during the test period is subnormal, the water diuresis may be described as "impaired."

[6] "Inactivation," as used in this text, applies to the disappearance of antidiuretic hormone from the circulating blood stream as a result of destruction, excretion, or some combination of these processes.

Eisenmenger (2) has summarized some of the abnormalities of water metabolism in the patient with cirrhosis and ascites and their possible relationship to increased production of the antidiuretic hormone. Following the ingestion of sodium by the cirrhotic, the deficient excretion of this ion may result in such prolonged relative elevation of the serum sodium as to stimulate the secretion of ADH. Hyponatremia may then reflect the retention of water out of proportion to sodium, due to this increased production of ADH. Another possible cause of overproduction of ADH in the presence of a dilute extracellular fluid may be the stimulation of antidiuretic activity by a "volume receptor" in response to a decreased volume of cerebral blood and extracellular fluid. In support of such a hypothetical mechanism Eisenmenger adduces the work of Lewis, Buie, Sevier and Harrison (17), Leaf and Mamby (18), and Strauss, Davis, Rosenbaum, and Rossmeisl (19).

On the other hand, a report of prolonged antidiuresis following the intravenous administration of Pitressin to three patients in congestive heart failure (20) suggests a decreased rate of "inactivation" of Pitressin, and/or an increased sensitivity of the end-organ, the renal distal tubule.

Increased neurohypophysial antidiuretic activity resulting in water retention has also been suggested by the occurrence of hyponatremia in some patients with congestive heart failure (21–24). Stead (25) has discussed the objections to such a concept.

The specific aims of the present experiments have been to: (1) study water diuresis following intravenous hydration with 5 per cent glucose in water in patients with congestive heart failure; (2) observe the effects of Pitressin administered intravenously during the course of continuous intravenous hydration; and (3) compare the responses of these cardiac patients with those demonstrated previously by patients with cirrhosis of the liver, and by control subjects.

EXPERIMENTAL

Water diuresis following intravenous hydration with 5 per cent glucose in water was studied in 10 patients suffering from congestive heart failure. In six of these the effect of single intravenous doses of Pitressin (0.57 mU per Kg.), administered during continued intravenous hydration, was also determined. Similar studies were performed on a patient with cirrhosis of the liver associated with ascites and edema (R. A.), in addition to those on hepatic patients reported previously (14). Table I presents a brief resumé of the clinical features of these experimental patients. The ten cardiacs and J. C., a cirrhotic, were on a low-salt regimen (500 mg. sodium per day); all other experimental subjects were on a regular intake of salt.

Each subject was in a fasting state for 15 hours. Prior to the beginning of the experiment, one glass of water was taken orally every half hour for two hours. An intravenous infusion of 5 per cent glucose in water (average flow of 10 cc. per minute) was then started and continued throughout the entire experiment. When the rate of urine flow reached and maintained a plateau for three collection periods (each approximately fifteen minutes long), 0.57 mU per Kg. of Pitressin was injected rapidly into the infusion tubing near the needle.

The urinary bladder was washed out with distilled water and air at the end of each collection period (about fifteen minutes in length) through an indwelling six-holed catheter.

Blood specimens were obtained at the beginning of an experiment, at the height of the diuresis, at the height of antidiuresis, and at the end of the procedure.

The calculations and definitions employed in the present paper are the same as those used previously (14). The "per cent inhibition" =

$$\frac{\text{Expected diuresis minus observed diuresis} \times 100}{\text{Expected diuresis}}.$$

The "expected diuresis" is the urine flow that would have been obtained if the initial rate, observed just before the Pitressin was injected, had been maintained throughout the period of Pitressin effect.

METHODS

Hematocrit determinations on heparinized blood were performed by centrifuging in Wintrobe tubes at 2,500 rpm. for 30 minutes. Serum and urinary creatinine chromogen concentrations were measured by Peters' modification of the Folin method (26); urinary sodium and potassium, and serum potassium and sodium were measured with an internal standard Perkin 52A flame photometer; serum sodium, as indicated, was measured by the Butler-Tuthill gravimetric method (27); chloride was determined by the Van Slyke-Hiller modification of Sendroy's iodometric method (28); and urinary glucose measured by the method of Benedict (29). Standard methods of statistical analysis were employed (30).

RESULTS

A. Endogenous creatinine chromogen clearance

The endogenous creatinine chromogen clearance averaged 67 ± 21 cc. per min. in the cardiac patients; 85 ± 9 cc. per min. in those with hepatic disease; and 80 ± 25 cc. per min. for the controls (mean of 99.7 cc. for the non-arthritic controls,

TABLE I

*The effect of intravenous Pitressin on the renal excretion of water in congestive heart failure and in liver cirrhosis**

Patient	Age	Sex	Diagnosis	AS†	P.ED.†	Ccr‡ *cc./min.*	Hydration: Time to reach P.D. *(min.)*	Hydration: P.D. *cc./min.*	Antidiuresis: % inhibition	Antidiuresis: Duration of pit. effect *(min.)*	Peak antidiuresis *cc./min.*
Cardiacs:											
S. K.	67	M.	ASHD	+	+	42	106	7.3	—§	112§	0.9
L. R.	22	F.	RHD	+	0	—	109	9.5	—§	60§	0.4
H. D.	40	F.	RHD	0	0	88	77	8.6	52	58	1.1
E. S.	44	F.	RHD	0	+	73	48	6.9	52	63	0.5
G. M.	37	F.	RHD	+	+	55	121	5.2	48	57	0.7
F. C.	41	F.	RHD	0	+	96	89	11.6	54	57	0.6
A. P.	34	M.	RHD	0	0	75	107	8.4	—	—	—
T. N.	50	F.	RHD	+	0	43	139	2.1	—	—	—
P. Z.	59	F.	RHD	+	+	—	141	7.1	—	—	—
S. P.	60	F.	HHD, ASHD	+	+	—	54	0.6	—	—	—
$\bar{x}$ =						67	99	6.7	52	69	0.7
						±21	±32	±3.3	±3.4	±22	±0.3
Liver Disease:‖											
M. W.	42	F.	Cirrhosis	±	+	89	92	13.1	54	70	1.8
J. L.	50	M.	Infectious hepatitis	0	0	82	127	11.8	57	79	1.0
T. R.	52	F.	Cirrhosis	+	+	98	60	14.7	52	56	1.4
R. A.	52	M.	Cirrhosis	+	+	73	65	6.9	60	80	0.7
J. C.	56	M.	Cirrhosis	+	0	85	99	12.9	45	69	2.1
$\bar{x}$ =						85	88	11.9	54	71	1.4
						±9	±27	±2.9	±5.5	±10	±0.6
Controls:‖											
D. S.	51	F.	Rheumatoid arthritis	0	0	66	68	13.5	54	60	1.8
R. D.	19	F.	Arthritis	0	0	—	123	12.2	55	60	2.1
J. P.	62	M.	A-V Aneurysm rt. post. orbital ves.	0	0	80	57	11.1	53	60	0.9
E. N.	40	M.	Silicosis	0	0	124	80	14.6	47	81	0.2
M. I.	52	F.	Arthritis	0	0	54	95	11.1	62	69	1.2
S. G.	39	M.	Complete ht. block	0	0	95	72	14.7	64	65	1.1
E. B.	50	F.	Arthritis	0	0	64	133	6.8	54	52	0.7
$\bar{x}$ =						80	85	12.0	56	64	1.1
						±25	±32	±2.6	±5.6	±9.2	±0.7

* The numbers in parentheses after the initials of the following cardiac subjects indicate the days preceding the experimental run on which a mercurial diuretic was administered; a number followed by an asterisk denotes the length of the hospital stay preceding the investigation, and represents a known minimum period during which no mercurials were administered: S. K. (31*); L. R. (3*); H. D. (28, 17, 15, 8); E. S. (3*); G. M. (8*); F. C. (12, 3); A. P. (10*); T. N. (30, 28, 26, 12); P. Z. (10*); and S. P. (19, 14, 8).

The absence or presence of ascites and peripheral edema is denoted by 0 or +, respectively.

† AS—Ascites; P.ED.—Peripheral Edema.

‡ Ccr—Endogenous Creatinine Clearance.

§ Urine flow did not return to pre-Pitressin level during experimental period.

Abbreviations: P.D.—Peak Diuresis; ASHD—Arteriosclerotic heart disease; RHD—Rheumatic heart disease; HHD—Hypertensive heart disease.

‖ Data on these patients (except for R. A.) have been presented previously.

Note: All ten cardiac patients were in congestive heart failure.

and 61 cc. per min. for the patients with arthritis) (Table I).

B. *Excretion of water*

Following the onset of the intravenous infusion of 5 per cent glucose in water, the time required for attainment of peak diuresis did not differ significantly, statistically, in any of the three groups of subjects: 99 ± 32, 88 ± 27, and 85 ± 32 minutes for the cardiac, hepatic, and control subjects, respectively (Table I, Figure 1); P = 0.36 for cardiacs *vs.* controls, and 0.53 for cardiacs *vs.* cirrhotics. On the other hand, the level of peak diuresis was significantly lower in the patients suffering from congestive heart failure (6.7 ± 3.3 cc. per min.) than in the controls (12.0 ± 2.6 cc. per min.) (P = 0.003) or in the hepatic patients (11.9 ± 2.9 cc. per min.) (P = 0.009) (Table I,

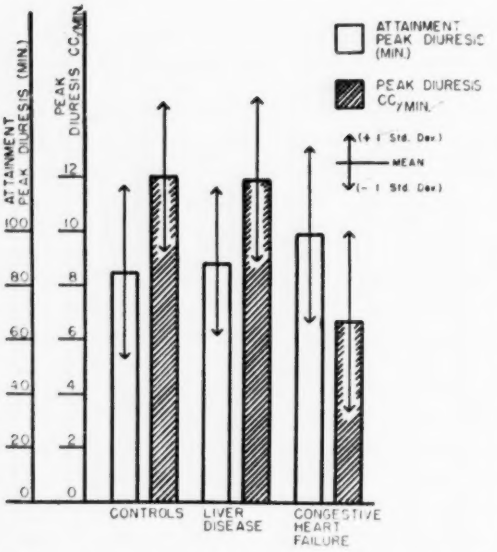

FIG. 1. THE TIME REQUIRED TO ATTAIN PEAK DIURESIS FOLLOWING THE ONSET OF INTRAVENOUS HYDRATION WITH 5 PER CENT GLUCOSE IN WATER (10 CC. PER MIN.) AND THE LEVEL OF PEAK DIURESIS ATTAINED

Figure 1). Four of the cardiacs were not given Pitressin; in two of these (T. N. and S. P.) the urine flow during the hydration period did not reach rates deemed high enough, while restiveness in the other two (A. P. and P. Z.) precluded complete study.

Of the six cardiac patients in whom the effects of intravenous Pitressin (0.57 mU per Kg.) were studied, five demonstrated responses which were similar to those reported for controls and for hepatic patients. The "per cent inhibition" and duration of Pitressin effect could not be calculated for L. R. because the experiment was not run long enough to permit the urine flow to return to pre-Pitressin levels. Thus, for the four cardiac patients, H. D., E. S., G. M., and F. C., the duration of Pitressin antidiuresis averaged 58.6 ± 3.4 min. ($P = 0.20$ for cardiacs *vs.* controls, and 0.12 for cardiac *vs.* hepatic patients). The "per cent inhibition" of diuresis in the patients with congestive heart failure averaged 51.7 ± 3.4 per cent ($P = 0.16$ for cardiacs *vs.* controls, and 0.55 for cardiac *vs.* hepatic patients) (Figure 2). The other cardiac patient, S. K., demonstrated an enhanced Pitressin antidiuresis (Figure 3).

C. *Serum electrolytes*

Table II presents the effect of Pitressin on the serum electrolytes. Pitressin had no statistically significant effect on the serum concentrations of sodium, chloride or potassium in the cardiac patients (except G. M.).

Although the initial concentrations of sodium and chloride in the serum tended to be lower in the cardiac patients than in the other groups, the only statistically significant difference was that observed in the chlorides.

D. *Urinary electrolytes*

The renal excretion (mEq. per min.) of sodium and chloride during the hydration period (H) as well as during antidiuresis was significantly lower in the cardiac than in the controls (for chloride, $P = 0.006$ and for sodium, $P = 0.004$). If one of the cirrhotics, M. W., is excluded, there is no statistically significant difference in the urinary excretion of sodium and chloride between the cardiac and hepatic patients. The excretion of potassium in the urine (mEq. per min.) during the hydration period was the same for the three groups of experimental subjects.

Table III and Figure 4 demonstrate that Pitressin had no statistically significant effect on the minute output of urinary sodium, chloride or potassium, either in the cardiac patients or in the other two groups.

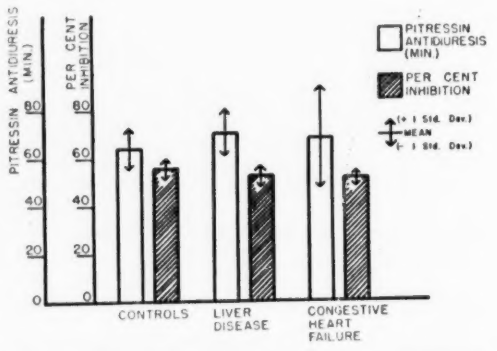

FIG. 2. THE DURATION OF PITRESSIN ANTIDIURESIS AND THE "PER CENT INHIBITION OF DIURESIS" FOLLOWING THE INTRAVENOUS ADMINISTRATION OF 0.57 MU PER KG. PITRESSIN TO CONTINUOUSLY-HYDRATED (INTRAVENOUS 5 PER CENT GLUCOSE IN WATER) SUBJECTS

DISCUSSION

A. *Endogenous Creatinine Chromogen Clearance*

The low clearance values in the arthritic controls resulted from a diminished urinary excretion (mg. per min.), while the plasma concentrations were normal. This may be related to the abnormal glycine metabolism reported in rheumatoid arthritis (31), resulting possibly in a diminished synthesis of creatinine and creatine. Another possibility is that there may have been an increased creatinuria (as a result of muscular atrophy) with concomitantly diminished creatininuria.

B. *Excretion of Water*

1. *Time required for attainment of peak diuresis.*

Burn and Grewal (32) believe that the time required for the attainment of peak diuresis may be a measure of the ADH present (in the kidney and circulating in the blood) when the water was ingested. If this is correct, then the demonstration (that the time required for maximum diuresis in the patients with heart disease was not significantly different, statistically, from that observed in liver disease or in control subjects) would tend to exclude hypersecretion of ADH in our patients suffering from either of these diseases.

It is difficult to reconcile such apparent lack of hypersecretion and the absence of deficient inactivation of the hormone with the reported increases of an antidiuretic substance (ADS) in the urine of such patients as well as in the serum of cirrhotic patients, unless one were to assume that ADS is indeed not derived from the neurohypophysial antidiuretic hormone.

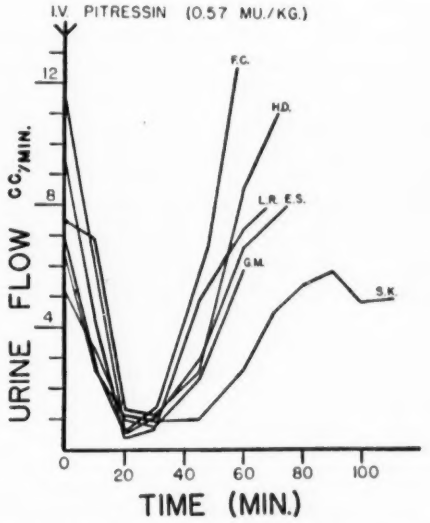

FIG. 3. THE EFFECT OF INTRAVENOUS PITRESSIN (0.57 MU PER KG.) ON THE URINE FLOW IN CONTINUOUSLY-HYDRATED (INTRAVENOUS 5 PER CENT GLUCOSE IN WATER) PATIENTS SUFFERING FROM CONGESTIVE HEART FAILURE

S. K. is the only subject who showed an enhanced antidiuretic effect.

2. *Peak rate of diuresis*

While the peak rate of diuresis attained in the patients with cirrhosis was not significantly different statistically from that observed in the control subjects, the peak rate in the cardiac patients

TABLE II

Effect of Pitressin on serum electrolytes

	Initial			Diuresis			Antidiuresis			Final		
	Na mEq./L.	Cl mEq./L.	K mEq./L.	Na mEq./L.	Cl mEq./L.	K mEq./L.	Na mEq./L.	Cl mEq./L.	K mEq./L.	Na mEq./L.	Cl mEq./L.	K mEq./L.
Cardiacs:												
S. K.	121.9	90.7	5.6	120.2	90.0	5.1	121.6	90.4	5.4	121.5	90.2	5.3
L. R.	130.0	98.7	4.8	130.3	98.8	3.8	125.8	97.2	4.0	—	—	—
H. D.	131.5	92.8	4.0	128.6	91.0	3.8	129.1	92.1	3.6	129.1	93.4	3.2
E. S.	137.7	96.0	3.9	131.4	96.9	3.5	137.7	96.3	3.7	135.0	96.1	3.4
G. M.	136.2	95.2	3.6	131.1	91.2	3.6	125.9	91.2	3.5	128.3	90.2	3.4
F. C.	136.4	97.3	4.3	137.8	97.4	4.1	—	—	—	—	—	—
$\bar{x}$	132.3	95.1	4.4									
Liver Disease:												
$\bar{x}$	138.0	104.0	3.9									
Controls:												
$\bar{x}$	137.4	103.7	4.6									

$\bar{x}$—Mean.

TABLE III

Urinary electrolyte excretion during hydration and Pitressin antidiuresis

Patients	Hydration (H)					Antidiuresis (A)					(H) Minus (A)			
	Time min.	Urine flow cc./min.	Cl mEq./min.	Na mEq./min.	K mEq./min.	Time min.	Urine flow cc./min.	Cl mEq./min.	Na mEq./min.	K mEq./min.	Urine flow cc./min.	Cl mEq./min.	Na mEq./min.	K mEq./min.
Cardiacs:														
S. K.	112	4.3	.017	.003	.063	112*	3.3	.014	.004	.044	+1.0	+.003	−.001	−.019
L. R.	68	6.8	.015	.003	.022	68*	4.3	.023	.003	.018	+2.5	−.008	.000	+.004
H. D.	58	7.0	.012	.011	.045	58	4.1	.015	.009	.052	+2.9	−.003	+.002	−.007
E. S.	63	6.4	.012	.013	.023	63	3.3	.009	.007	.024	+3.1	+.003	+.006	−.001
G. M.	57	4.4	.012	.013	.017	57	2.7	.010	.009	.017	+1.7	+.002	+.004	.000
F. C.	57	9.9	.019	.007	.079	57	5.2	.016	.007	.064	+4.7	+.003	.000	+.015
t											6.5	.94	1.6	1.2
p											.0013	.41	.17	.29
Liver Disease:														
t											4.8	.73	1.9	.15
p											.0085	.52	.13	.93
Controls:														
t											9.7	.40	.90	1.6
p											.0001	.70	.40	.16

* Urine flow did not return to pre-Pitressin level during experimental period.

"Pre-Pitressin control period" (H) is that portion of the intravenous hydration period which just precedes the Pitressin injection, and which is equal in time to the "antidiuresis period" for a given patient.

$t = \frac{\bar{d}}{S_{\bar{d}}}$, where $\bar{d}$ = difference between the means and $S_{\bar{d}} = S_{\bar{x}}$ for (H) minus (A).

p = probability if p = 1.0, then the observed difference is entirely a chance occurrence.
if p = 0.0, then there is no element of chance.

was significantly lower than in the other two groups. Since the time required for the attainment of peak diuresis was normal and Pitressin was handled normally by the cardiac patients (with one exception), it is unlikely that abnormalities of ADH metabolism were responsible for the diminished peak diuresis.

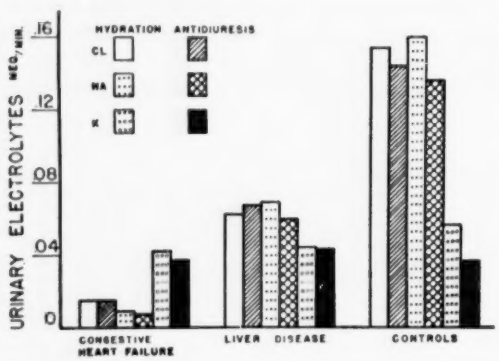

FIG. 4. THE EFFECT OF INTRAVENOUS PITRESSIN (0.57 MU PER KG.) ON THE AVERAGE URINARY EXCRETION OF ELECTROLYTES IN CONGESTIVE HEART FAILURE, LIVER DISEASE AND CONTROL SUBJECTS

Statistical analysis showed no significant difference among the three groups of patients with respect to the effect of Pitressin on the urinary electrolytes.

Among the differences noted in the cardiac patients from the other two groups were the diminished urinary excretion of sodium and a tendency for the endogenous creatinine clearances to be lower. Since the renal tubular reabsorption of sodium may be associated with that of water, increased reabsorption of the sodium ion might explain a smaller excretion of water. All else being equal, the level of peak diuresis is a function of the glomerular filtration rate, decreases in which would tend to diminish the level of peak diuresis attained (33).

C. *Pitressin Antidiuresis*

1. *Excretion of water*

As mentioned previously, Sinclair-Smith and co-workers (20) observed prolonged antidiuresis in three patients with congestive heart failure. In the present experiments, only one of the cardiacs (S. K.) demonstrated such prolongation. The data available do not definitely explain this unique behavior. Of the patients with congestive heart failure to whom Pitressin was administered, S. K. had the lowest endogenous creatinine clearance (42 cc. per min.), the lowest concentrations of sodium (121.9 mEq. per L.) and chloride (90.7

mEq. per L.) and the highest concentration of potassium (5.6 mEq. per L.) in the serum. The possibility exists that these alterations in renal hemodynamics and serum electrolytes might be responsible for the prolonged antidiuretic response to Pitressin.

The normal responses of the older cardiac patients and of the patients with hepatic disease to Pitressin tend to exclude a decreased rate of inactivation (in physiological dosage) and also tend to eliminate the possibility of increased renal tubular sensitivity to the exogenous hormone. As we have discussed previously (14), to the extent that the inactivating mechanisms for exogenous Pitressin and endogenous ADH are similar, these results exclude a decreased rate of inactivation of ADH (in physiological amounts) in these disease states.

2. Serum electrolytes

While Pitressin (in the dosage used) tended to lower the serum sodium in the hepatic patients, it had no consistent effect on the concentrations of sodium, chloride, or potassium in the serum of patients with congestive heart failure. To the extent that the physiological effects of Pitressin and ADH are similar, one cannot ascribe the hyponatremia, that has been observed in some patients with congestive heart failure, to ADH circulating in normal amounts.

3. Urinary electrolytes

The urinary excretion of sodium and chloride was decreased in the patients with congestive heart failure. Part of this diminished excretion might be ascribed to their low intake of salt. Pitressin had no statistically significant effect on the urinary excretion of sodium, chloride, or potassium, and this parallels our previous findings in control subjects and in those with hepatic cirrhosis.

D. *Relationship to These Studies to the Question of Possible Over-production of ADH in Hepatic and Cardiac Disease*

We have referred to the reported increases of ADS in the urine of patients with congestive heart failure and in the serum and urine of those with hepatic cirrhosis. Our experiments were designed to determine whether such increases might result, in part, from a decreased rate of inactivation of ADH, and this seems to be excluded. Overproduction of ADH might then explain these increases of ADS, if ADS is derived from ADH. Since it has not been proved that ADS is indeed derived from ADH (34), overproduction of ADH in these disease states cannot be postulated with any degree of certainty at this time. Finally, as mentioned earlier in this discussion, the normal time for attainment of peak diuresis observed in both groups of patients might possibly be used to exclude hypersecretion of the neurohypophysial antidiuretic hormone in our patients with congestive heart failure as well as those with hepatic cirrhosis. Since the present series is relatively small, further study may reveal patients with more definite evidence of continued hypersecretion of ADH.

SUMMARY AND CONCLUSIONS

1. Water diuresis following intravenous hydration with 5 per cent glucose in water at 10 cc. per min. was studied in ten patients suffering from congestive heart failure. The time required for peak diuresis to be reached was 99.1 ± 32.1 minutes, which did not differ significantly from the control. However, the average level of peak diuresis, 6.7 ± 3.3 cc. per min., differed significantly from that observed in control subjects, 12.0 ± 2.6 cc. per min., and in hepatic patients, 11.0 ± 2.9 cc. per min.

2. Pitressin antidiuresis was studied in six of the cardiac patients. In four of these, the per cent inhibition of diuresis and the duration of Pitressin effect did not differ significantly from the control, but in one of the cardiacs there was a significantly enhanced Pitressin antidiuresis.

3. Pitressin, in the dosage used, did not affect the concentrations of serum sodium, chloride or potassium in the patients with congestive heart failure, but tended to lower the serum sodium of the hepatic patients.

4. To the extent that the "inactivating" mechanisms for Pitressin and the antidiuretic hormone (ADH) are similar, our demonstration of normal rates of inactivation of Pitressin (in physiological dosage) in patients with hepatic cirrhosis or congestive heart failure tends to exclude a decreased rate of inactivation of ADH (in physiological amounts) in these diseases.

5. If the reported increases of antidiuretic substance (ADS) in the blood and urine of such patients represent increased amounts of endogenous ADH, then the exclusion of a decreased rate of inactivation of ADH would tend to favor increased production as an explanation for the findings. However, evidence available at present does not definitely establish the conclusion that such continued overproduction of ADH exists.

ACKNOWLEDGMENTS

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STUDIES IN STEROID METABOLISM. XIX. THE α-KETOSTEROID EXCRETION PATTERN IN NORMAL MALES[1]

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The urinary steroids afford a means for evaluation of the production of steroid hormones by the adrenal and gonads. Any alteration in either rate of type of hormone production by the glands resulting from specific disease or from hormonal therapy therefore must be manifest by qualitative or quantitative change in the excretion of steroid hormone metabolites. In order to delineate the composition of these metabolites in normal healthy subjects, the present investigation describing the α-ketosteroid excretion pattern was undertaken.

MATERIALS AND METHODS

Twenty normal males, ranging in age from 21 to 76, were studied. Of this number, eighteen were White and one was Negro; Subject D31 was a Nisei. Subjects C21 and M21 were normal males hospitalized at the Peter Bent Brigham Hospital, Boston, Massachusetts, for the purpose of studying some of the metabolic effects of oral Cortisone and intravenous ACTH; Subjects K31, R32, R63, C64, M65, and M72 were subjects of similar study at the Baltimore City Hospitals, Baltimore, Maryland. Only control periods of these eight subjects are reported here. The remainder of the subjects are, or were, employees of this Institute, working and in apparent good health. The details of urine collection and handling in this laboratory have been reported previously (1). Urine was collected for periods ranging from 1 to 198 days, although only four subjects had urine collections longer than 20 days.

Hydrolysis: During the course of the study, and with the aim of providing more reliable techniques, several hydrolytic procedures have been used. A short description of the different methods of hydrolysis follows.

Method A: Stage 1. The urine was adjusted to pH 1 with concentrated sulfuric acid. To this solution, concentrated sulfuric acid equaling 5 per cent of the original volume was added. The urine was then heated under reflux for one-half hour. After cooling, the urine was continuously extracted with ether for 24 hours.

Stage 2. The ethereal extract was washed several times with saturated sodium bicarbonate solution.

Stage 3. The other solution from Stage 2 was washed several times with 2N sodium hydroxide.

Stage 4. The ether solution from Stage 3 was washed with 10 per cent hydrochloric acid and with water, until the washings were neutral. Aqueous and acid extracts were discarded. The ether was removed under diminished pressure and the residue obtained was referred to as the "crude neutral steroidal fraction."

Stage 5. "Crude acidic" and "crude phenolic" fractions were obtained from the alkali soluble material from Stages 2 and 3 by methods reported previously (1) and stored for future work. "Crude acidic" and "crude phenolic" fractions were prepared from alkali soluble extracts in Methods B, C, and D as well.

Method B: Stage 1. The urine was adjusted to pH 1 with concentrated sulfuric acid and continuously extracted with ether for 48 hours.

Stage 2. The ether solution was extracted with saturated sodium bicarbonate as in Method A, Stage 2. The ether solution remaining was processed to the "crude neutral steroidal fraction" (as in Method A, Stages 3 and 4).

Stage 3. The sodium bicarbonate wash from Stage 2 was acidified to pH 1 with sulfuric acid, 5 per cent by volume of concentrated sulfuric acid was added, and the solution heated under reflux for one-half hour. After cooling, it was continuously extracted with ether for 24 hours, processed as in Method A, Stages 2, 3, and 4, and the "crude neutral fraction" was obtained.

Stage 4. The spent urine after Stage 1 was acidified with 5 per cent of its volume of concentrated sulfuric acid,

[1] The authors gratefully acknowledge the assistance of grants from the American Cancer Society (on recommendation of the Committee on Growth of the National Research Council), the Commonwealth Fund, the Anna Fuller Fund, the Lillia Babbitt Hyde Foundation, and the National Cancer Institute of the National Institutes of Health of the United States Public Health Service.

[2] Konrad Dobriner died March 10, 1952.

[3] So far as we are aware, the interpretation of the data and the conclusions drawn are in essential agreement with the views held by the late Dr. Dobriner.

[4] Research Fellow of the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council.

heated under reflux for one-half hour and extracted with ether for 24 hours. This ether extract was treated as in Method A, Stages 2, 3, and 4, and the "crude neutral fraction" was obtained.

This method of hydrolysis yielded three "crude neutral fractions"; one was obtained by "mild" hydrolysis and two were obtained by "vigorous" acid hydrolysis.

Method C: Stage 1. The initial acidification and extraction of urine and spent urine was that followed in Method 2, Stages 1, 2, and 4.

Stage 2. The sodium bicarbonate wash (obtained as in Method 2, Stage 3) was adjusted to pH 5 with concentrated sulfuric acid, and then 10 per cent by volume of sodium acetate buffer at pH 5 and 300,000 units per liter of solution of beef liver β-glucuronidase were added. The solution was incubated at 37° for 48 hours. After incubation, the solution was adjusted to pH 1 with concentrated sulfuric acid and was extracted with ether for 48 hours. The "crude neutral fraction" from this ether extract was obtained as in Method A, Stages 2, 3, and 4.

Stage 3. The sodium bicarbonate wash from Stage 2, above, was processed and another "crude neutral fraction" was obtained (as in Method A, Stages 1, 2, 3, and 4).

Stage 4. The aqueous residue after ether extraction from Stage 2 was treated as in Method A, Stages 1, 2, 3, and 4, and a final "crude neutral fraction" was obtained.

This hydrolytic method yielded five crude neutral fractions; two of these were obtained by "mild" hydrolysis, and three were obtained by "vigorous" acid hydrolysis.

Method D: Stage 1. The urine was adjusted to pH 5 with concentrated sulfuric acid and 10 per cent by volume of sodium acetate buffer at pH 5 and 300,000 units per liter of solution of beef liver β-glucuronidase were added. The solution was incubated at 37° for 48 hours. After incubation, the urine was adjusted to pH 1 with concentrated sulfuric acid and extracted with ether for 48 hours. The "crude neutral fraction" was obtained from this ethereal extract as in Method A, Stages 2, 3, and 4.

Stage 2. The spent urine after Stage 1, was treated as in Method A, Stages 1, 2, 3, and 4 and the "crude neutral fraction" was obtained.

Stage 3. The sodium bicarbonate wash from Stage 1 was treated as in Method C, Stage 2, and the ethereal extract was obtained.

Stage 4. The ethereal extract obtained in Stage 3, above, was treated as in Method A, Stages 2, 3, and 4, and the "crude neutral fraction" obtained.

Stage 5. The sodium bicarbonate extract from Stage 4 was treated as in Method A, Stages 1, 2, 3, and 4, and the "crude neutral fraction" obtained.

Stage 6. The aqueous residue after ether extraction from Stage 3, above, was treated as in Method A, Stages 1, 2, 3, and 4, and a "crude neutral fraction" obtained.

This method of hydrolysis yielded five crude neutral fractions; two of these fractions were obtained under conditions of "mild" hydrolysis and three were obtained by "vigorous" acid hydrolysis.

Fractionation: The "crude neutral steroid fractions" obtained after these various hydrolytic procedures were separated into ketonic and non-ketonic fractions by means of Girard's "Reagent T" and into α and β-ketosteroid fractions by precipitation with digitonin. These methods have been described in detail (1). The ketosteroid fractions were then separated by chromatography on alumina or silica gel columns. When Method B was used, the three "crude neutral steroid fractions" were separately fractionated and chromatographed. The sum of the values obtained for individual steroids from each fraction was used for the pattern. When Method C or D was used, the "crude neutral steroid fractions" obtained after "mild" hydrolysis were combined prior to fractionation and chromatography. Similar but separate treatment was accorded the "crude neutral steroid fractions" obtained after "vigorous" acid hydrolysis. After separation into ketonic and non-ketonic material, the ketonic portion from "mildly" hydrolyzed extracts was partitioned between benzene and water according to the procedure of Mason (2). Separation into α and β subfractions with digitonin was done on the benzene soluble material and only the α-ketosteroids were included in the pattern. The vigorously hydrolyzed fractions were not partitioned between benzene and water. The α-ketosteroids from the two types of hydrolytic procedures were sometimes combined prior to chromatography but more frequently separate chromatograms were made and the sum of the individual values was used for the pattern.

The water extracts, obtained from benzene-water partition (Methods C and D) were chromatographed separately, after acetylation with pyridine and acetic anhydride at room temperature. The water extraction procedure is a recent addition to the methodology and relatively few urine extracts were processed through this stage. Therefore the values for substances normally encountered in this fraction which are lacking in Table I and Figure 2 were omitted because the fraction was unavailable.

Each individual fraction from the chromatogram was analyzed by infrared spectrometry and quantitation of the separated steroids was done by the Zimmerman reaction (as modified by Callow, Callow, Emmens, and Stroud [3, 4] and Talbot, Butler, MacLachlan, and Jones [5]). Since ketosteroids differ somewhat in chromogenic value, appropriately determined factors were used to correct the color values from milligrams of dehydroisoandrosterone, used as the standard, to milligrams of the actual compound determined in the chromatographic fraction. Fractions obtained from the water soluble extracts were analyzed by ultraviolet spectrometry for compounds containing an α,β-unsaturated ketone (Compounds E and F) and by the Zimmerman reaction with appropriate factors, for saturated compounds (tetrahydro E and F).

Traces of 3β-etiocholanolone often appear in α-ketonic fractions. Though this compound is quantitatively precipitated by digitonin when pure, this is not the case with urinary extracts. Thus the figures reported do not estimate total daily excretion of this steroid. The separation of androstanedione and etiocholanedione from each other and from 3β-etiocholanolone is incomplete and quantitation is distorted by impurities, eluted from this portion of

TABLE I

The urinary steroid excretion pattern in normal males

Compounds	*Milligrams per 24 hours*									
Total C_{19} 11-oxy steroids	2.8	2.8	.6	2.6	2.6	3.4	.8	1.2	2.1	3.6
Total C_{19} 11-desoxy steroids	14.8	12.2	4.0	10.4	7.6	8.4	4.3	3.6	5.7	12.9
Compounds E & F								.3	.3	
Tetrahydro E & F								.4	1.0	
17-Hydroxypregnanolone	—	—	—	—	—	—	—	—	—	—
11-Ketoandrosterone	—	—	tr.	—	.1	.6	—	tr.	—	tr.
11-Ketoetiocholanolone	.8	1.1	.5	1.3	1.0	1.2	.7	.2	.6	1.8
11-Hydroxyetiocholanolone	.4	—	—	—	—	—	—	—	—	—
11-Hydroxyandrosterone	1.6	1.7	.1	1.3	1.5	1.6	.1	1.0	1.5	1.8
Etiocholanolone	7.6	6.0	2.7	6.9	3.3	4.7	2.3	1.8	3.1	7.6
Androsterone	7.0	6.2	1.0	3.0	3.0	3.0	1.6	1.8	2.5	4.3
"Preg. group"	—	—	.4	—	1.8	—	.7	—	—	1.0
3β-Etiocholanolone	—	—	.1	—	.2	—	.1	—	—	.3
Etiocholanedione	.1	tr.	.1	tr.	.2	tr.	.2	tr.	tr.	.4
Androstanedione	.1	tr.	.1	.5	.3	.7	.1	tr.	.1	.3
% β of $\alpha + \beta$	4.5	7.7	4.4	10.1	6.0	7.8	3.4	20.2	6.1	9.7
β-Ketonic KS	1.1	1.6	.4	2.0	.8	1.3	.3	1.9	.7	2.3
α-Ketonic KS	23.5	19.3	8.6	17.8	12.5	15.4	8.6	7.5	10.8	21.4
Ketonic KS	24.3	23.7	9.8	22.6	15.5	17.1	11.2	12.9	13.1	26.1
Crude KS	28.8	29.8	27.4	22.8	21.7	19.4	15.3	20.2	22.5	29.0
Hydrolysis	D	D	B	A	A	A	A	C	C	A
Collection days	9	9	**	**	**	21	**	15	15	33
Patient-age	M21	C21	B21	Y27	N30	G30	D31	K31	R32	D35

* Hydrolyzed according to Method B, eliminating Stage 3.
** Average of values of replicate patterns.

TABLE I—*Continued*

Compounds	*Milligrams per 24 hours*									
Total C_{19} 11-oxy steroids	1.1	2.4	1.3	1.4	1.4	3.2	.8	.9	4.1	.5
Total C_{19} 11-desoxy steroids	10.9	7.6	7.2	4.1	4.9	4.2	1.7	2.5	6.2	.8
Compounds E & F							.4			
Tetrahydro E & F							.6			
17-Hydroxypregnanolone	—	—	—	—	—	—	—	—	—	—
11-Ketoandrosterone	tr.	.8	.3	tr.	—	—	—	tr.	—	tr.
11-Ketoetiocholanolone	1.0	1.5	.4	.7	.5	1.2	.3	.6	.9	.5
11-Hydroxyetiocholanolone	—	—	—	—	—	1.0	—	—	.3	tr.
11-Hydroxyandrosterone	.1	.1	.6	.7	.9	1.0	.5	.3	2.9	tr.
Etiocholanolone	8.0	3.7	4.8	2.6	2.5	2.1	1.2	1.7	4.0	.5
Androsterone	2.7	3.4	1.6	1.1	2.4	2.1	.5	.4	2.2	.2
"Preg. group"	.8	2.2	1.7	1.5	—	—	—	.7	—	.5
3β-Etiocholanolone	.1	.1	.2	.1	—	—	tr.	tr.	—	.1
Etiocholanedione	.1	tr.	.3	.1	tr.	tr.	tr.	.2	tr.	tr.
Androstanedione	tr.	.4	.3	.2	tr.	tr.	tr.	.2	tr.	tr
% β of $\alpha + \beta$	1.4	2.4	6.2	4.3	16.2	—	2.1	1.8	4.1	2.4
β-Ketonic KS	.2	.3	1.0	.4	1.6	tr.	.1	.1	.5	.1
α-Ketonic KS	14.5	12.4	15.2	8.9	8.3	7.8	4.7	5.6	11.8	4.1
Ketonic KS	18.6	16.3	19.2	10.7	11.8	9.7	5.2	6.0	13.9	5.3
Crude KS	21.7	16.1	54.8	35.3	23.0	17.4	15.1	9.5	21.0	14.0
Hydrolysis	A	B*	A	A	C	C	C	A	C	A
Collection days	**	38	4	4	15	9	15	5	9	5
Patient-age	T42	W50	M51	F54	R63	C64	M65	L66	M72	M76

the chromatographic columns. In those hydrolytic procedures employing vigorous acid hydrolysis a variable proportion of the compounds 11-hydroxyandrosterone and 11-hydroxyetiocholanolone were isolated as their $\Delta^{9,11}$-unsaturated analogs. This occurred especially when hydrolytic methods A and B were used, though very small amounts of these unsaturated analogs sometimes are encountered when the enzymatic methods are employed, probably as a result of dehydration on the adsorbent surface.

In all cases it is evident that the sum of isolated and identified steroids does not equal that of the original α-ketonic value. There is no evidence that this unidentified fraction contains anything more than trace amounts of ketosteroids, and the discrepancy between the "crude" and the isolated ketosteroids very probably results from the elimination of non-specific chromogens.

Terminology: The following terminology has been employed in this laboratory—and is employed in this paper—for brevity and convenience in reference: ***dehydroisoandro-***

sterone for 3β-hydroxy-Δ⁵-androstene-17-one; *etiocholanedione* for etiocholane-3,17-dione; *androstanedione* for androstane-3,17-dione; *androsterone* for 3α-hydroxyandrostane-17-one; *etiocholanolone* for 3α-hydroxyetiocholane-17-one; *11-hydroxyetiocholanolone* for 3α,11β-dihydroxyetiocholane-17-one; *11-hydroxyandrosterone* for 3α, 11β-dihydroxyandrostane-17-one; *11-ketoetiocholanolone* for 3α-hydroxyetiocholane-11,17-dione; *11-ketoandrosterone* for 3α-hydroxyandrostane-11,17-dione; *17-hydroxypregnanolone* for 3α,17α-dihydroxypregnane-20-one; *tetrahydro E for* 3α,17α,21-trihydroxypregnane-11,20-dione; *tetrahydro F* for 3α,11β,17α,21-tetrahydroxypregnane-20-one; *Compound E* for 17α,21-dihydroxy-Δ⁴-pregnene-3,11,20-trione; *Compound F* for 11β,17α,21-trihydroxy-Δ⁴-pregnene-3,20-dione. *Preg. group* refers to a specific group of eluents from alumina columns appearing immediately before androsterone and characterized by infrared spectrometry as a 20-ketosteroid containing a hydroxyl group; pregnanolone has previously been isolated from a pool of this material. This group sometimes contains, in addition, an unidentified 17-ketosteroid. Because the color factor for this group is high (6.5) the quantitative values may be seriously in error. In the graphs, 11-hydroxy and 17-hydroxy have been abbreviated to 11-OH and 17-OH.

RESULTS

The quantitative data on the α-ketonic steroid pattern in male urine are found in Table I. Subjects are listed by initial and in ascending order of age, with hydrolytic method, length of collection, crude steroid data and the steroid excretion pattern listed in appropriate sections of the Table. The high values for crude ketosteroid excretion are the result of the relatively complete extraction from urine of both ketones and non-ketonic alcohols as well as non-specific chromogens. The

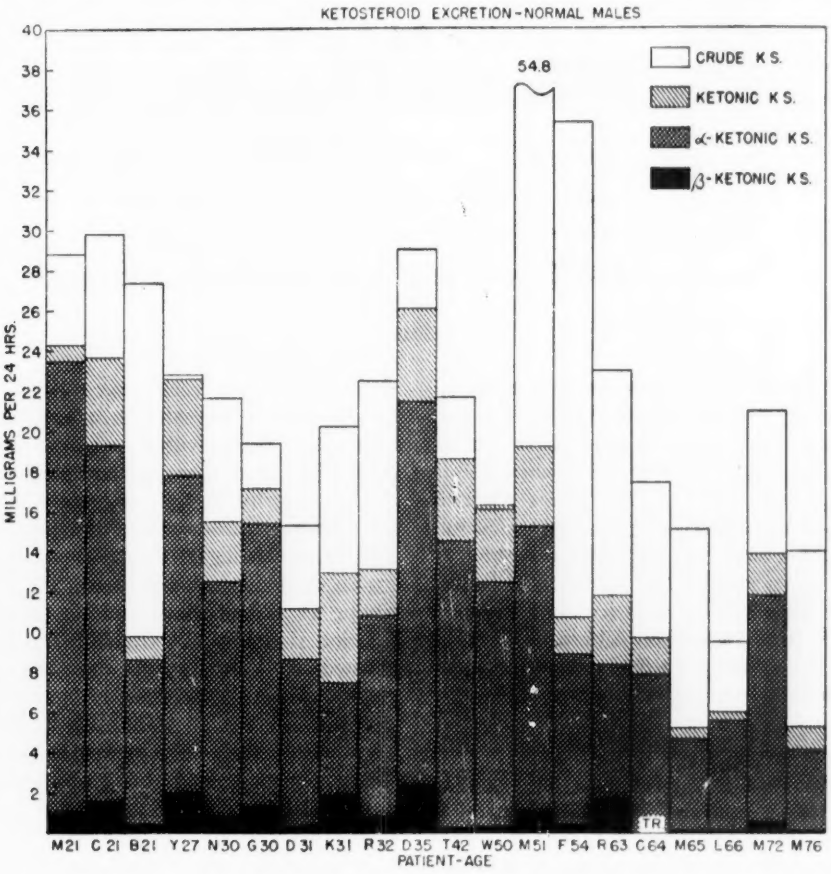

FIGURE 1

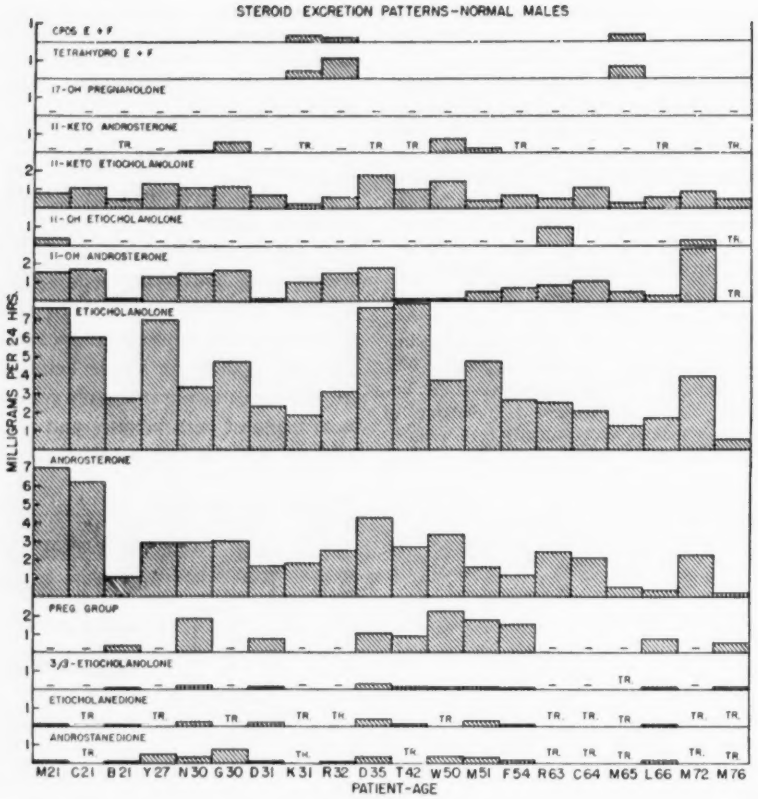

FIGURE 2

values for the ketonic fraction are more truly representative of the ketosteroids and are similar to the data of other investigators on ketosteroid excretion in normal subjects. In subjects B21, Y27, N30, D31 and T42, the figures represent mean values of several patterns run on each subject (two, two, three, four, and three, respectively).

Figure 1 is a graphic representation of patient, age, and crude ketosteroid excretion; Figure 2 depicts the steroid excretion pattern on the same subjects, listing all isolated and identified components in milligrams per twenty-four hours.

DISCUSSION

The actual studies reported have been in progress over twelve years and during this time various alterations and improvements in technique have been introduced. Despite the progressive change in methodology, it is apparent that a fairly consistent pattern of results has emerged. The α-ketosteroids of normal men are sufficiently descriptive of the individual to permit certain generalizations about hormones and their metabolism.

The majority of the compounds identified in the α-ketonic fraction can be grouped as follows: 1) Steroids having 19 carbon atoms and containing an oxygen function at carbon 11; 2) Steroids having 19 carbon atoms and lacking an oxygen function at carbon 11. Group 1, the C_{19} 11-oxygenated metabolites, quantitatively the small component, may account for from 10 to as much as 45 per cent of the total identifiable material in the α-ketonic fraction. The hormonal precursors of the compounds found in this group are almost unquestionably products of the adrenal cortex, since there is no evidence to suggest that 11 oxygenation is a capacity of any other tissue (excepting perhaps placenta, which does not come into consider-

ation in this series). The precursor steroid or steroids must be substances with a ketone or hydroxyl function at C-11, and probably in addition, an oxygen function at C-17. Of the known adrenal hormones, hydrocortisone clearly meets the qualifications. Since studies of adrenal vein blood (6, 7), perfusion of surviving gland (8), and direct isolation (9) are in agreement that this hormone is one of the principal products of adrenocortical activity, it may be presumed that urinary steroids of Group 1 are derived chiefly from this precursor.

There is a large individual variation from subject to subject in the daily excretion of C_{19} compounds with an 11-oxygen function. It is evident that this range of values is not attributable to age, as shown in Figure 3. Though modal values differ somewhat, men in the age group 50 years and above excrete C_{19} 11-oxygenated steroids in approximately the same amounts as men below 50 years. Thus, it would appear that adrenal function

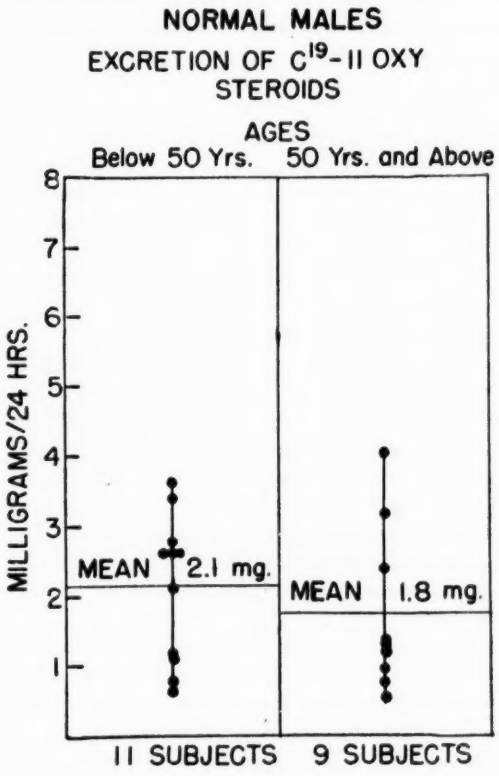

FIGURE 3

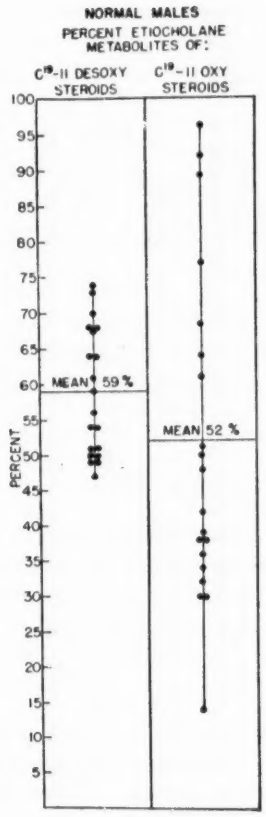

FIGURE 4

under the conditions studied, is not appreciably altered with age.

Two steroids, 11-hydroxyandrosterone and 11-ketoetiocholanolone are the principal, and to all practical purposes, the only components of Group 1. Values for excretion of 11-hydroxyandrosterone range from trace amounts to 2.9 mg. per 24 hours with mean value of 1.0 mg. per 24 hours. Values for excretion of 11-ketoetiocholanolone range from .2 mg. per 24 hours to 1.8 mg. per 24 hours with a mean value of .8 mg. per 24 hours. There is no constant relationship of these two substances to one another in the various subjects studied. In ten of the subjects 11-hydroxyandrosterone was present in greater amounts, and in seven, 11-ketoetiocholanolone was the larger component. Figure 4 depicts the percentage of the Group 1 metabolites found in the form of derivatives of the parent hy-

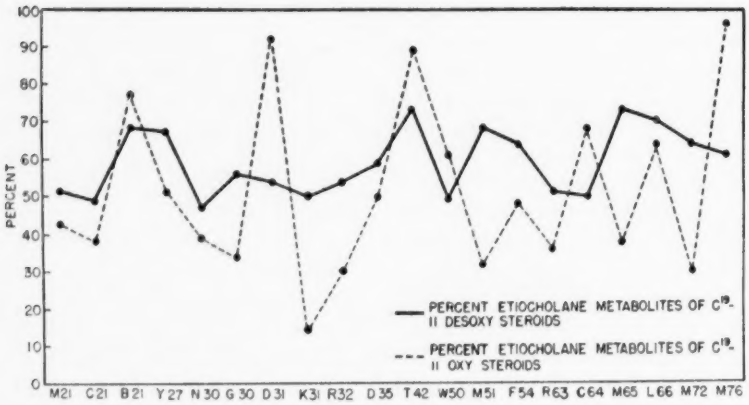

FIGURE 5

drocarbon etiocholane (Rings A : B-cis). It is evident that there is extremely wide variation in the per cent of metabolites excreted as this isomer. This variation appears unrelated to either age, total daily output of C_{19} 11-oxy steroids, or the per cent of C_{19} 11-desoxy steroids excreted as this isomer (Figure 5). The significance of this variation in the type of A : B ring juncture effected by different subjects is unknown at present.

Two other steroids are found in this group, though with considerably less regularity and in very much smaller amounts. (11-ketoandrosterone was found in trace amounts in seven, and in larger amounts in four of the twenty subjects.) Its presence does not appear related to age or the level of any other transformation product. 11-hydroxyetiocholanolone was found in the urine of four normal subjects. In three of these, it was present in considerably greater than trace amounts (M21, C64, M72) ; three out of the four subjects in whose urine it has been identified are 64 years of age or over (C64, M72, M76). The presence of 11-hydroxyetiocholanolone in urine has previously been related with a high degree of significance to the existence of malignancy in the subject studied (10–12). Moreover, three subjects previously selected as normal controls and whose urine showed the presence of greater than trace amounts of 11-hydroxyetiocholanolone, each developed and was treated for a malignant tumor while the present program of studies was in progress. It is known that 11-hydroxyetiocholanolone may appear in the urine of patients who have received ACTH as therapy or for study purposes (13). However, coincident with any appreciable rise in 11-hydroxyetiocholanolone excretion, one finds a considerable rise in urinary levels of 11-hydroxyandrosterone and 11-ketoetiocholanolone. Thus it would not be reasonable to ascribe the presence of 11-hydroxyetiocholanolone in these normals, and in cancer patients, to a markedly increased secretion of all adrenal hormones, since an associated high level of other adrenocortical metabolites was not evident. It is believed, therefore, that the tendency to production of relatively large amounts of the metabolite 11-hydroxyetiocholanolone reflects a metabolic variant generally present in patients with neoplasia and conceivably in a segment of the normal population whose predisposition towards development of cancer is greater than average.

Group 2, the C_{19} 11-desoxysteroids, is uniformly the larger component of the α-ketonic fraction. The major precursor, in males, of the metabolites in this group is undoubtedly testosterone of gonadal origin. It is equally certain that the adrenal cortex secretes a steroid, or group of steroids, whose metabolic transformation products include C_{19} 11-desoxy compounds. The adrenal contribution to the compounds in this group, however, is normally low (14).

Individual excretion values of the steroids in Group 2, *i.e.*, the C_{19} 11-desoxy steroids, are capable of wide variation as can be seen in Table I and range from .8 mg. per 24 hours to 14.8 mg. per 24 hours. It is evident that while there is a large overlap of values, on the average, men 50 years of

age and over excrete smaller amounts of C_{19} 11-desoxy steroids in their urine than men under 50 as shown in Figure 6. That age alone is not the sole influential factor is evident by striking individual variation in the excretion of this group of substances. These differences in the excretion of 11-desoxy steroids by various individuals may well reflect changes in vigor in the broadest meaning of the term and the diversity of values may well suggest the heterogeneity of a segment of a normal population.

Etiocholanolone and androsterone together make up the great bulk of the steroids found in the C_{19} 11-desoxy group. That there is wide individual variation in the excretion of these two compounds by different subjects is evident from Figure 6. The tendency toward reduced excretion of these two metabolites with age explains the decreased excretion as a whole of Group 2 (C_{19} 11-desoxy) steroids with age, since the other constituents of this group are relatively minor in amount. Mean value for excretion of etiocholanolone in men under 50 years is 4.9 mg. per 24 hours and for men 50 years and above it is 2.6 mg. per 24 hours. Mean value for excretion of androsterone for these two groups is 3.3 and 1.5 mg. per 24 hours, respectively.

A few subjects excrete etiocholanolone and androsterone in approximately a 1 : 1 ratio. In the majority of men, however, etiocholanolone is excreted in greater amount than androsterone. Calculation of the mean etiocholanolone/androsterone ratio of the group under 50 (1.7/1) and the group 50 years and over (2.2/1) suggests that the metabolites of the etiocholane type are relatively greater in the older age group. The overlap is considerable, however, and the difference is not statistically significant.

Unlike their 11-oxygenated analogs, there seems to be a high order of constancy in the tranformation of C_{19} 11-desoxy precursors to metabolites having a particular type of fusion of the A : B rings of the steroid nucleus. It is apparent from Figure 4 that despite a very wide variation in total output of C_{19} 11-desoxy metabolites, the per cent of these metabolites, excreted as derivatives of etiocholane varies within a narrow range. That this constancy is unrelated to age, or to the per cent of C_{19} 11-oxygenated etiocholane metabolites is clear from Figure 5. Because of this lack of relationship, it is suggested that the reduction of the α,β-unsaturation in 11-oxy and 11-desoxy precursors with the formation of a particular isomer may have a specific significance in terms of the physiologic action of hormonal precursors.

In addition to etiocholanolone and androsterone, trace amounts of androstanedione and etiocholanedione are found in all urines; 3β-etiocholanolone is found in trace amounts infrequently. There is a suggestion that the sum total of the "diones" may be higher in those subjects whose etiocholanolone and androsterone output is high, but again exceptions, such as Subject M21 and C21 are apparent. The identification of these three steroids is technically complicated, as has been indicated under "Methods." No discussion of the "preg. group" is warranted because of its inhomogeneity and irregular detection in the chromatographic separation. 17α-hydroxypregnanolone has not

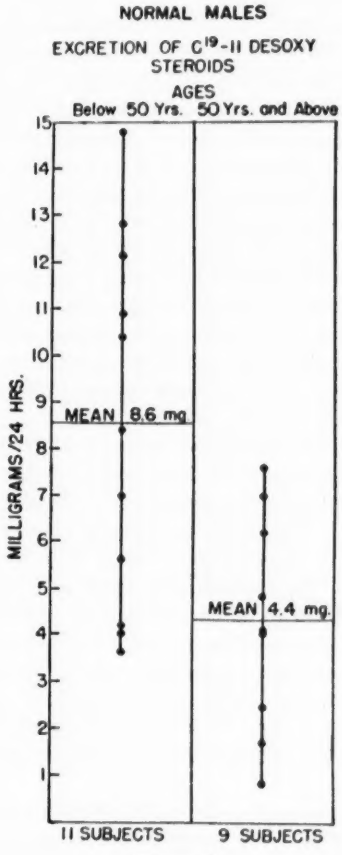

FIGURE 6

TABLE II

Mean α-ketosteroid excretion pattern

Compounds	Men below 50	Men 50 and above
C_{19} 11-desoxy Compounds	8.6	4.4
C_{19} 11-oxy Compounds	2.1	1.8
11-Ketoetiocholanolone	.9	.7
11-Hydroxyandrosterone	1.1	.8
Etiocholanolone	4.9	2.6
Androsterone	3.3	1.5
Etiocholanedione	trace	trace
Androstanedione	trace	trace
% β of $\alpha + \beta$	7.4	4.4
β-Ketosteroids	1.1	.5
α-Ketosteroids	14.5	8.8
Ketonic Ketosteroids	17.7	10.9
Crude Ketosteroids	23.5	22.9
Cases	11	9

been identified in normal male urine. The significance of its presence in disease states is thus further established. Isolation of compounds E and F and their "tetrahydro" derivatives has been effected in only three subjects. As has been noted previously, the tetrahydro compounds are excreted in greater amounts than their unsaturated precursors. Studies on patients receiving cortisone or ACTH therapy indicate this to be the pattern of excretion at widely varying levels of precursor steroids (15).

There is a great deal of published data on crude ketosteroid excretion in normals and in various disease states, so that these data need not be dealt with extensively. Two points appear worthy of mention. First, the association of ketosteroid output with age is more clearly apparent only as finer fractionation of the ketosteroids is made. The clear distinction between changes in 11-desoxy and 11-oxygenated steroid output can be demonstrated only after extensive separation of crude steroid fractions. The limits to interpretation of crude data are clearly evident. Second, it is worthy of note that the β-ketosteroid fraction, which has been ascribed to adrenal precursors, undergoes a selective decrease with age, not associated with any apparent alteration of the other adrenal metabolites that have been studied. Elimination of the unusually high values in Subjects K32 and R61 accentuates this selective decrease in the β-ketosteroid fraction.

Mean values of all the crude and pattern data are presented in Table II where the subjects are divided into two groups, men below the age of 50, and men 50 years and above in age; 11-hydroxyetiocholanolone is not included for reasons discussed above. Though the limitations of calculating "average" patterns for at least these two groups are apparent from the wide individual variations present, for general comparative purposes this breakdown is useful in that it permits the interpretation of older and less refined data in the light of the information obtained by the extensive and intensive analytical methods employed in the present study.

SUMMARY

The individual components of the neutral α-ketosteroid fraction from urine were determined in twenty normal males ranging in age from 21 to 76 years. After preliminary separation, the steroids were isolated by chromatography and were identified by infrared spectrometry. The principal components in a quantitative sense were 3α-hydroxyetiocholane-11,17-dione, 3α,11β-dihydroxyandrostane-17-one, etiocholanolone, and androsterone. Other compounds were present in small or trace amounts.

The presence of an oxygen atom at carbon-11 of the steroid nucleus permitted distinction of metabolites primarily derived from adrenal hormones. By means of this criterion, it was concluded that adrenal function was essentially constant during the decades studied, whereas the level of metabolites, derived primarily from gonadal precursors, diminished in the older group. Despite this trend, there were variations with individual subjects within age groups so that it would appear the determinants of hormone production and metabolism are peculiar to the subject rather than a consequence of chronological age. The significance of these results in the interpretation of hormone physiology was discussed.

ACKNOWLEDGMENTS

We are grateful to Dr. George Thorn for making available to us the urine on Subjects C21 and B21, and to Dr. Nathan Shock for making available the urine on Subjects K31, R32, R63, C64, M65, and M72.

The authors wish to express their appreciation to the large group of devoted Research Assistants and Technicians who made much of the work possible. The routine chemical and chromatographic separations were carried out by a group under the supervision of Madeleine Stokem and Ruth Jandorek. The colorimetric analyses

were under the supervision of Alga Teager. The infrared spectrometry was under the supervision of Friederike Herling.

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STUDIES IN STEROID METABOLISM. XX. THE REPRODUCIBILITY OF THE URINARY STEROID PATTERN IN HUMANS[1]

By KONRAD DOBRINER[2]

THE DATA WERE ASSEMBLED AND THE MANUSCRIPT PREPARED BY:[3]

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(Submitted for publication April 6, 1953; accepted May 29, 1953)

The most important problem that confronts an investigator concerned with a physiological process is the reproducibility of both the technique and the phenomenon under study. It is implicit in the investigation of steroid excretion that a given subject will exhibit a high order of reproducibility of his steroid pattern under comparable physiological conditions. Expressed in another way, steroid hormone production and metabolism as measured by the urinary excretion products should be reasonably constant for a normal individual. In the course of extensive studies of steroid metabolism in progress in this Institute, it has been possible to examine this problem and to demonstrate that in several subjects of different age and habitus the pattern of steroid excretion of a particular individual is reproducible with a high degree of precision.

METHODS

The methods employed in this laboratory for the fractionation, isolation, and identification of steroid metabolites have been published (1); the hydrolytic methods referred to are described in paper number XIX (2) in this series.

RESULTS

Replicate patterns, determined over varying periods of time, have been studied in nine normal male subjects; the initial, followed by the age, is descriptive of the subject. The steroid patterns in these subjects are listed in Table I. Crude ketosteroid data and the values for isolated compounds are listed in appropriate portions of the Table. Four compounds representing the major components of the α-ketonic fraction are listed; androsterone, etiocholanolone, 11-hydroxyandrosterone and 11-ketoetiocholanolone.

Subject C21: Patterns 1 and 2 were determined three months apart. Between these two periods, a course of intravenous ACTH was given for another study. A week intervened between the end of the ACTH period and the beginning of the urine collection for Pattern 2. There was no evidence of residual effect of the ACTH as determined by the metabolites studied.

Subject M21: Patterns 1 and 2 were determined under the same conditions and time limits as Subject C21.

Subject B21: Patterns 1 and 2 were obtained from successive long-term collections over a period of 10 months.

Subject Y27: Patterns 1 and 2 were determined on long-term urine collections separated by an interval of two years.

Subject N30: Patterns 1, 2, and 3 were determined within a period of two months.

Subject D31: Patterns 1, 2, 3, and 4 were determined within a five month period at approximately monthly intervals.

Subject K31: The details of Patterns 1 and 2 in this subject are similar to those of Subject R32.

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[2] Konrad Dobriner died March 10, 1952.

[3] So far as we are aware, the interpretation of the data and the conclusions drawn are in essential agreement with the views held by the late Dr. Dobriner.

[4] Research Fellow of the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council.

TABLE I

Replicate steroid patterns in normal males

Compounds	Mg. per 24 hrs.										
11-Ketoetiocholanolone	1.1	1.0	.8	1.5	.5	.5	1.8	.9	.6	1.2	1.4
11-OH Androsterone	1.7	1.6	1.6	1.6	.1	.2	1.3	1.2	2.3	1.3	1.1
Etiocholanolone	6.0	5.9	7.6	6.3	2.9	2.5	7.9	6.0	3.2	3.7	3.1
Androsterone	6.2	7.6	7.0	5.9	1.0	1.1	3.5	2.5	2.5	2.7	3.6
% β of $\alpha + \beta$	7.7	12.4	4.5	2.6	2.6	5.9	8.3	12.2	6.5	5.0	6.6
β-Ketonic KS	1.6	3.1	1.1	.6	.2	.6	1.8	2.2	.8	.7	.9
α-Ketonic KS	19.3	22.2	23.5	22.1	7.6	9.5	19.8	15.8	11.6	13.2	12.7
Ketonic KS	23.7	24.9	24.3	26.9	7.7	11.8	23.1	22.0	15.3	15.0	16.3
Crude KS	29.8	31.5	28.8	32.7	20.3	34.4	23.3	22.2	19.1	24.6	21.3
Hydrolysis	D	D	D	D	B	B	A	A	A	A	A
Pattern number	1	2	1	2	1	2	1	2	1	2	3
Patient-Age	C21		M21*		B21		Y27			N30	

* This subject excreted 11-OH etiocholanolone in the following amounts during control periods: Period 1—.4 mg. and Period 2—.6 mg.

TABLE I—*Continued*

Compounds	Mg. per 24 hrs.										
11-Ketoetiocholanolone	.6	.8	.8	.7	.2	.3	.6	.8	.6	1.1	1.3
11-OH Androsterone	tr.	tr.	.3	tr.	1.0	.9	1.5	.9	.3	tr.	tr.
Etiocholanolone	2.9	2.1	2.1	2.0	1.8	1.9	3.1	3.8	4.9	8.9	10.1
Androsterone	1.7	1.3	1.2	2.0	1.8	1.9	2.5	1.7	1.8	2.9	3.5
% β of $\alpha + \beta$	3.2	3.7	3.9	2.8	20.2	13.0	6.1	4.5	1.6	1.3	1.2
β-Ketonic KS	.3	.4	.3	.2	1.9	.9	.7	.4	.2	.2	.2
α-Ketonic KS	9.2	10.5	7.5	7.0	7.5	6.3	.7	8.6	12.4	14.8	16.3
Ketonic KS	11.3	14.4	10.0	9.1	12.9	8.6	13.1	10.9	15.6	19.9	20.3
Crude KS	18.6	17.5	14.4	10.8	20.2	15.1	22.5	22.0	20.4	21.6	23.2
Hydrolysis	A	A	A	A	C	C	C	C	A	A	A
Pattern number	1	2	3	4	1	2	1	2	1	2	3
Patient-Age	D31				K31		R32			T42	

Subject R32: Patterns 1 and 2 were determined three months apart. During the intervening period, a course of intramuscular injections of ACTH was given for another study. Pattern 2 followed the cessation of ACTH by an interval of two weeks.

Subject T42: Patterns 1, 2 and 3 were determined at one month intervals.

DISCUSSION

These data are presented initially in this series for two purposes: 1) To demonstrate the degree of reproducibility of techniques employed in the extensive studies to be presented later. 2) To show clearly that without the stimulus of acute or chronic pathologic or metabolic change, as exemplified by disease, hormonal therapy, etc., steroid excretion tends to vary only within narrow limits. The determinants of these limits are as yet unknown.

SUMMARY

Data are presented that indicate a high order of constancy in the production, metabolism, and excretion of steroid hormones as determined by isolation and quantitation of individual hormonal metabolites.

ACKNOWLEDGMENTS

The authors wish to express their appreciation to the large group of devoted Research Assistants and Technicians who made much of the work possible. The routine chemical and chromatographic separations were carried out by a group under the supervision of Madeleine Stokem and Ruth Jandorek. The colorimetric analyses were under the supervision of Olga Teager. The infrared spectrometry was under the supervision of Friederike Herling.

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THE LOCAL BLOOD FLOW IN HUMAN BONE MARROW IN LEUKEMIA AND NEOPLASTIC DISEASES AS DETERMINED BY THE CLEARANCE RATE OF RADIOIODIDE (I^{131}) [1]

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Although considerable information has been obtained on the rate of blood flow in many organs (1), few data have been reported on the blood flow in human or animal bone marrow (2). The neglect of this aspect of marrow physiology is probably due to the technical difficulties encountered in gaining access to the blood vessels of this diffuse organ in the intact animal or man.

The extensive application of the radioisotope clearance technique described by Kety (3, 4) for the estimation of the regional circulation in muscle, liver, subcutaneous tissues, brain tissue, and skin (4–10) suggested its use as a clinically applicable method for the evaluation of the effective local blood flow in the human bone marrow. This method is based on the premise that if a diffusible tracer substance is introduced into a tissue its clearance or removal is a function of all possible factors contributing to the local blood flow at the site of injection.

In the present study, the relative rates of clearance of I^{131} from the bone marrow were determined in patients with various malignant diseases and leukemia. The radioiodide was injected into the bone marrow through a standard bone marrow needle, and the rate of disappearance of the isotope was measured with a Geiger-Mueller counter. In addition, simultaneous determinations of I^{131} clearance rates from the gastrocnemius muscle were obtained for comparing the clearance rates obtained in the bone marrow with those found in an anatomically and physiologically different vascular bed.

MATERIALS AND METHODS

Clinical material: Forty-three patients with a variety of malignant diseases and leukemia were studied (Table I). The patients were grouped into seven major groups as indicated in Table I. They varied in weig t from 28 to 91.6 kilo, and all had normal blood pressures except M. D. and E. H., whose pressures were 175/110 and 160/110, respectively (Table I). The subjects were studied under resting but not basal conditions. The environmental temperature varied from 20 to 25° C.

Marrow technique: The sternal marrow and the gastrocnemius muscle were studied simultaneously in each patient (Table I). In a few subjects the tibia and the ilium were employed. The iliac crest site was approximately two centimeters posterior to the anterior superior spine of the ilium and the tibia was studied at the tibial tubercle.

The marrow site was aseptically prepared and locally anesthetized with 2 per cent procaine infiltration of the skin, subcutaneous tissue and periosteum. A short Turkel needle was advanced into the bone with a rotary motion until the marrow cavity was entered. The radioiodide was then introduced into the marrow tissue by means of a tuberculin syringe with a No. 22 gage 4-inch needle inserted through and embedded well beyond the tip of the bone marrow needle. The I^{131}, as sodium iodide, was slowly injected, usually requiring one to two minutes. A slow rate of injection was used to minimize pain and to avoid a sudden increase of intramedullary pressure which conceivably might affect the rate of clearance of the I^{131}. By placing the point of the No. 22 gage needle well into the marrow and slowly injecting the radioactive material, it was possible to prevent skin contamination by reflux of marrow blood and I^{131} into the marrow needle. Following the injection of I^{131} both needles were withdrawn from the marrow and pressure was immeditely applied to prevent bleeding into the subcutaneous tissues and onto the skin. The puncture site was cleaned with alcohol to remove possible radio-

[1] Presented in part at the Annual Meeting of Western Section of the American Federation for Clinical Research, Carmel, California, January 24, 1952.

[2] Postgraduate Research Fellow, National Cancer Institute.

TABLE I

Summary of pertinent clinical and clearance data obtained from 43 patients

Patient	Diagnosis	Age	Sex	Hb *gm./100 ml.*	WBC *per cm.*	Marrow clearance I^{131}			Muscle clearance I^{131}			Ratio $\frac{\text{Marrow K}}{\text{Muscle K}}$
						Vol. *ml.*	T½ *min.*	K *% per min.*	Vol. *ml.*	T½ *min.*	K *% per min.*	
Non-leukemic diseases												
J. Mc.	Carcinoma lung	51	M	15.6	7,400	0.05*	15.6	4.44				
T. W.	Carcinoma kidney	43	M	10.2	8,800	0.70	7.8	8.88	0.4	9.3	7.45	1.19
M. D.	Carcinoma cervix	40	F	6.9	32,000	0.25	9.3	7.45	0.18	7.7	9.00	0.83
L. E.	Carcinoma breast	39	F	12.0	6,500	0.50*	11.8	5.87	1.20	12.7	5.46	1.07
A. B.	Sq. carcinoma tongue	59	M	10.6	12,400	0.30	5.50	12.6	0.15	7.0	9.90	1.27
A. L.	Melanoma	47	M	10.2	6,700	0.20*	12.5	5.54				
J. K.	Melanoma	63	M	13.3	4,200	0.15*	8.4	8.25				
E. H.	Fibrosarcoma	46	M	13.8	7,200	0.25	16.0	4.33	0.20	11.5	6.03	0.72
R. G.	Fibrosarcoma	42	M	11.6	10,550	0.80	7.7	9.00 7.37†	0.09	7.3	9.49 7.89†	0.95
Lymphoblastoma												
E. C.	Hodgkin's disease	12	F	11.1	5,400	0.40 0.40	8.5 8.4	8.15 8.25*	0.20	6.2	11.2	0.73
R. M.	Hodgkin's disease	24	M	5.0	4,700	0.25	24.2	2.86				
G. B.	Hodgkin's disease	27	M	4.9	6,100	0.30	7.2	9.63	0.12	7.5	9.24	1.04
J. H.	Lymphosarcoma	69	M	10.3	3,800	0.40*	12.7	5.46				
J. Z.	Lymphosarcoma	55	M	9.4	12,400	0.16*	20.5	3.38				
I. R.	Lymphosarcoma	23	M	14.3	9,400	0.80	6.8	10.20	0.40	8.5	8.15	1.25
G. H.	Mycosis fungoides	62	M	9.5	1,700	0.70	9.7	7.14 6.69†	0.40	6.7	10.3‡ 9.72†	0.69
Chronic lymphocytic leukemia												
Z. D.	Chronic lymphocytic	5	F	13.9	5,700	0.30	6.1	11.4	0.15	11.5	6.03	1.90
B. H.	Chronic lymphocytic	13	F	9.1	3,500	1.00	4.45	15.57	0.50	8.10	8.55	1.82
C. M.	Chronic lymphocytic	73	M	12.7	95,000	0.50	3.2	21.7	0.50	11.7	5.92	3.66
J. A.	Chronic lymphocytic	79	M	15.5	32,000	0.25	8.0	8.66	0.10	6.0	11.6	0.75
A. M.	Chronic lymphocytic	57	M	11.7	305,000	0.35	9.5	7.29	0.18	10.3	6.73	1.08
T. H.	Chronic lymphocytic	9	M	10.6	1,700	0.40*	4.7	14.7	0.15	3.6	19.3	0.76
H. P.	Chronic lymphocytic	63	F	13.8	500,000	0.35	8.01	8.60	0.12	5.68	12.2	0.70
D. P.	Chronic lymphocytic	65	M	7.6	321,000	0.90	8.75	7.92	0.35	11.3	6.16	1.29
B. B.	Chronic lymphocytic	75	M	8.2	177,000	0.50	3.7	18.7	0.25	14.0	4.95	3.78
J. V.	Chronic lymphocytic	16	M	6.3	800	0.85	3.7	18.7 13.34†			9.04†	

TABLE I—*Continued*

Patient	Diagnosis	Age	Sex	Hb gm./100 ml.	WBC per cm.	Marrow clearance I^{131}			Muscle clearance I^{131}			Ratio Marrow K / Muscle K
						Vol. ml.	T½ min.	K % per min.	Vol. ml.	T½ min.	K % per min.	
Chronic myelocytic leukemia												
M. M.	Chronic myelocytic	63	F	15.2	43,000	0.63	8.42	8.23	0.38	10.9	6.35	1.29
J. B.	Chronic myelocytic	49	F	13.8	194,000	0.25	5.5	12.6	0.10	4.5	15.4	0.82
H. L.	Chronic myelocytic	31	F	8.7	348,500	0.25	8.2	8.45	0.10	9.4	7.37	1.15
A. J.	Chronic myelocytic	41	M	8.0	168,000	0.40	3.4	20.4	0.20	4.0	17.3	1.18
W. F.	Chronic myelocytic	66	M	11.4	118,500	0.70	8.3	8.35	0.40	9.8	7.07	1.18
							9.0	7.70*				
J. S.	Chronic myelocytic	47	M	7.4	127,500	0.30	7.9	8.77	0.15	9.3	7.45	1.18
A. M.	Chronic myelocytic	71	F	9.9	130,000	0.60	14.5	4.78	0.20	11.4	6.08	0.79
								9.77†			9.57†	
Acute leukemia												
E. J.	Monoblastic	31	M	9.8	67,000	0.45	4.4	15.8	0.30	15.0	4.62	3 42
A. C.	Monoblastic	44	M	12.2	2,000	0.20	3.5	19.8	0.10	12.9	5.37	3.79
I. D.	Lymphoblastic	54	M	9.0	40,500	0.25	3.4	20.4				
R. W.	Lymphoblastic	32	F	4.1	110,500	0.28*	5.5	12.6				
R. R.	Myeloblastic	17	M	3.8	36,000	0.28*	4.4	15.8				
								16.9†			4.99†	
Multiple myeloma and monocytic leukemia												
N. P.	Multiple myeloma	69	M	8.5		0.25	4.5	15.40	0.10	7.50	9.24	1.67
G. V.	Multiple myeloma	59	M	8.2	5,700	0.90	4.25	16.30	0.35	5.75	12.10	1.35
G. C.	Multiple myeloma	55	F	11.7	3,600	0.15	17.5	3.96	0.05	8.70	7.97	0.50
V. D.	Multiple myeloma	49	F	9.0	5,100	0.25	4.5	15.00	—	—	—	—
R. J.	Monocytic leukemia	27	M	9.0	10,000	0.20	7.2	9.63*	0.08	7.40	9.36	1.03
J. M.	Monocytic leukemia	47	M	11.6	130,000	0.15	13.6	5.10	0.05	3.80	18.20	0.28

* Iliac crest marrow.
† Average for group.
‡ Deltoid muscle.

active contamination, and the wound was sealed with collodion.

Marrow was obtained for cytological examination at the end of counting procedure in most instances. In a few patients, however, 0.1 to 0.2 cc. of marrow was obtained at the start of the study without demonstrable alteration in clearance. In the later instances care was taken to inject the I^{131} well beyond the point of the aspiration. Routine differential counts of the marrow samples were made with both supravital and Wright stained fixed preparations. Usually 200 cells were counted for the differentials.

In most patients, I^{131} clearance rates of the gastrocnemius muscle were simultaneously determined, except where noted (Table I). The radioiodide was injected into the belly of the muscle by means of a tuberculin syringe with a No. 26 gage 1½ inch hypodermic needle.

Physiologic and pharmacologic studies designed to demonstrate the effect on I^{131} clearance of altered systemic circulatory dynamics were carried out in a few subjects. These included exercise, phlebotomy and the intravenous injection of Paredrine[3] and of Bistrium bromide (Squibb).[4]

Determination of radioactivity: From 100 to 400 micrograms of iodide and from 10 to 30 microcuries of I^{131} were used in all determinations. The radioiodide was made up in isotonic phosphate buffer, pH 7.5, solution containing carrier potassium iodide. The volume of radioiodide injected per patient is shown in Table I. Surface counting was carried out with a lead-shielded (1.25 cm. thick) Victoreen or Tracerlab mica end-window counting tube mounted on an adjustable stand. The window thickness of the tubes varied from 3.5 to 1.8 mg. per $cm.^2$. The window-to-tissue distance was 0.5 cm. In addition, two layers of gauze were interposed between the counting tube and the skin over the marrow site. The radiation detected in the marrow under these conditions of geometry is predominantly gamma radiation. The Geiger-Mueller tubes were coupled to two conventional scalers, one tube being used for the marrow study and the other for the muscle determination. Each injection site was counted for a total of 1,024 counts, every 30 to 60 seconds. The counting was carried out for fifteen to thirty minutes after injection. In all cases the counts were greater than ten times background.

Failure to obtain a decrease in counting rate with respect to time was indicative of gross skin contamination by radioiodide. The high efficiency of our tube for beta radiation and the absence of a physiological mechanism for removal of I^{131} from the skin provided an extremely sensitive measure of skin contamination. Where skin contamination occurred, an attempt was made to resume counting after careful cleansing with 70 per cent alcohol. Data were rejected if counting could not be started within three minutes after the injection of I^{131}. Data were also rejected if a single exponential decrease did not occur within the first 15 to 30 minutes after injection. Skin contamination not sufficient to obscure marrow clearance was manifested by failure to obtain a single exponential decrease, since the I^{131} on the skin would contribute an essentially constant number of counts to the counting rate during the time of the study. Skin contamination did not occur with the muscle studies since the I^{131} was injected into the muscle from a lateral position. This enabled counting to be done over skin that was out of the puncture site.

Studies were made to ascertain whether the radioactivity being measured was actually in the bone marrow and that the clearance curves were not significantly influenced by extravasation of minute amounts of blood containing radioiodide into the subcutaneous tissues. Marrow clearances were made employing aluminum-shielded (1.96 mg. per $cm.^2$) Geiger tubes. Counting was continued until the clearance curve was established, after which the aluminum shield was removed and the curve again determined. The clearance curves obtained in this manner were not significantly different, indicating that the radioiodide was injected into the bone marrow and that practically no contamination of the subcutaneous tissue occurs under the conditions of the technique employed here. The error that results from such extravasation would decrease the clearance rate but would not detract from the positive findings reported here.

Calculations: The activity in counts per second was plotted against time on semilogarithmic paper. In all cases studied, the results could be described as a single, decreasing exponential function. While other studies (8) have indicated that the disappearance curve is a complex function, our studies were based on the initial component which was logarithmic and extended through the first 15 to 30 minutes. In a few cases other components developed later. The present studies were completed within 20 minutes in most cases. The results are expressed both as half-time in minutes ($T\frac{1}{2}$), and as clearance constant (K), which is the per cent administered radioiodide removed per minute, as obtained from the expression $K = \frac{0.693}{T\frac{1}{2}}$.

To determine the statistical significance of the clearance constant derived from the plot of the data on semilogarithmic paper, the regression line was determined and the standard deviation of the half-time of the regression line was calculated (Mather, K., Statistical analysis in biology, Chapter VIII. Interscience Publ. Inc., New York, 1947) in four representative patients for marrow and muscle. It can be seen that the experimental data fit an exponential function and that there is a significant difference between marrow clearances of different patients as well as significant differences between the marrow and muscle clearances in some of the patients (Figure 1).

Radiation dosage calculations: For calculation of the radiation received by the bone marrow, certain simplifying assumptions were made:

[3] B-4-hydroxyphenylisopropylamine.

[4] Hexamethonium bromide.

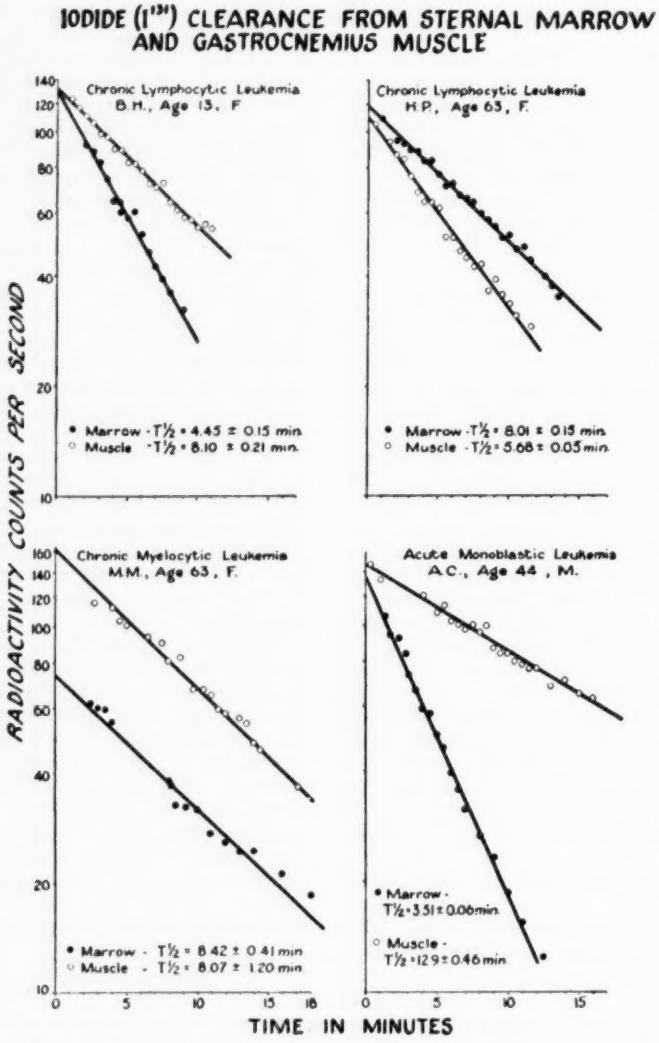
IODIDE (I^{131}) CLEARANCE FROM STERNAL MARROW AND GASTROCNEMIUS MUSCLE
Chronic Lymphocytic Leukemia
B.H., Age 13, F
• Marrow - T½ = 4.45 ± 0.15 min.
○ Muscle - T½ = 8.10 ± 0.21 min.
Chronic Lymphocytic Leukemia
H.P., Age 63, F.
• Marrow - T½ = 8.01 ± 0.15 min.
○ Muscle - T½ = 5.68 ± 0.05 min.
Chronic Myelocytic Leukemia
M.M., Age 63, F.
• Marrow - T½ = 8.42 ± 0.41 min.
○ Muscle - T½ = 8.07 ± 1.20 min.
Acute Monoblastic Leukemia
A.C., Age 44, M.
• Marrow - T½ = 3.51 ± 0.06 min.
○ Muscle - T½ = 12.9 ± 0.46 min.
RADIOACTIVITY COUNTS PER SECOND
TIME IN MINUTES

FIG. 1. COUNTS PER MINUTE OF I^{131} PLOTTED SEMILOGARITHMICALLY AGAINST TIME FOR STERNAL MARROWS AND GASTROCNEMIUS MUSCLES IN FOUR PATIENTS WITH LEUKEMIA

The half time of the regression line and the standard deviation of the half time are demonstrated.

(a) The depth of the sternal marrow cavity was assumed to be approximately one centimeter.

(b) Since less than 1 ml. of a solution containing less than 25 microcuries of I^{131} was administered, it was assumed that the greatest amount of radiation was confined to 1 $cm.^3$ of bone marrow tissue.

(c) In this volume the gamma ray energy contribution was considered to be negligible.

(d) No selective metabolic localization of I^{131} occurred in the bone marrow.

(e) The large dilution of I^{131} in the systemic iodide space, and the relatively rapid excretion of I^{131} (11), contributed only a small amount of the radioactivity to the marrow by recirculation and was considered insignificant.

(f) The effect of physical decay of I^{131} was considered

insignificant (half-life eight days) because of the much more rapid rate of removal of the I^{131} from the marrow site.

With these assumptions the irradiation to 1 gram of bone marrow from beta-particles with 30 microcuries of I^{131} is 0.260 rep. per min. (10). The total accumulated dose (complete removal of isotope from the site of injection) is 3.70 rep. Only about 0.07 per cent of the bone marrow receives this radiation, assuming a marrow volume of 1,400 cc. Clinically no evidence of hematopoietic dysfunction attributable to the radiation used in these studies could be detected.

The thyroid uptake of I^{131} resulting from marrow and muscle studies was determined in four patients at 6 and 24 hours, and was found to range from 15 to 25 per cent of the total administered dose. This represents 6 to 10 microcuries.

RESULTS

Forty-three patients with various neoplastic diseases, lymphoblastomas, and leukemia were studied. The half time ($T\frac{1}{2}$) and K values (per cent administered radioactivity removed per minute) for the bone marrow and muscle in each patient are presented in Tables I and II. Representing curves from four patients are shown in Figure 1. The distribution of the individual K values in the leukemic and non-leukemic patients is shown in Figure 2. In a patient with Hodgkin's disease (E. C.), simultaneous clearance studies on the sternum and iliac crest yielded almost identical results. The K values were closely grouped in the non-leukemic patients and in the patients with lymphoblastoma, with an average of 7.3 per cent I^{131} cleared per minute, ranging from 2.86 to 12.6 per cent. In the acute leukemias the average K value was 16.9 per cent per minute, ranging from 12.6 to 20.4 per cent, indicating that the injected iodide was removed from the marrow at a more rapid rate than in non-leukemic patients. In chronic lymphocytic leukemic patients there was a spread of K values from 7.29 per cent to 21.7 per cent per minute. Most of the K values for the myelocytic leukemia patients were in the range of the values obtained in the non-leukemic patients, 4.78 to 8.66 per cent per minute, except for patients J. B. and A. J. who had more rapid clearance rates. The two patients R. J. and J. M. with monocytic leukemia had clearance values of 9.63 per cent and 5.10 per cent, respectively. In four patients with multiple myeloma, three (N. P., G. V., and V. D.) had elevated K values, 15.4, 16.3, and 15.0 per cent, respectively, while the fourth patient (G. C.) had a low rate of 3.96 per cent.

There was no apparent correlation between the marrow clearance rates and the hemoglobin level, leucocyte count, weight, body temperature, and blood pressure (Figure 2). Although the volume of I^{131} in these experiments varied from 0.05 to 1.0 ml., there was no clearly demonstrable relationship between volume of injected I^{131} and clearance rate. Rapid clearance rates were obtained with both large and small volumes of I^{131}. The average volumes injected are indicated in Table I, and it can be seen that they did not vary more than 0.3 ml. for the marrow studies, and 0.2 ml. for the muscle studies.

In all the patients studied, the muscle K values were similar, approximately 8 to 10 per cent of the I^{131} removed per minute (Tables I and II). In the acute leukemia group, muscle clearances were obtained on only two patients, 4.62 and 5.37 per cent, respectively. One patient with monocytic leukemia had a muscle clearance of 18.2 per cent I^{131} removed per minute.

TABLE II

Averages of marrow and muscle clearance constants and ranges in 43 patients

	Bone marrow			Muscle		
	No. cases	Average K	Range	No. cases	Average K	Range
Non-leukemic	9	7.37	4.33–12.6	6	7.89	5.46– 9.90
Lymphoblastoma	7	6.69 14.06 Av. 7.03	2.86–10.2	4	9.72 17.61 Av. 8.31	8.15–10.3
Lymphatic leukemia	10	13.41	7.29–21.7	9	9.16	6.03–19.3
Myelocytic leukemia	7	10.3	4.78–20.4	9	9.65	6.08–17.3
Monocytic leukemia	2	7.36	5.10– 9.63	2	13.7	9.36–18.2
Multiple myeloma	4	13.4	3.96–16.3	3	9.77	7.97–12.1
Acute leukemia	5	16.9	12.6 –20.4	2	4.16	4.62– 4.70

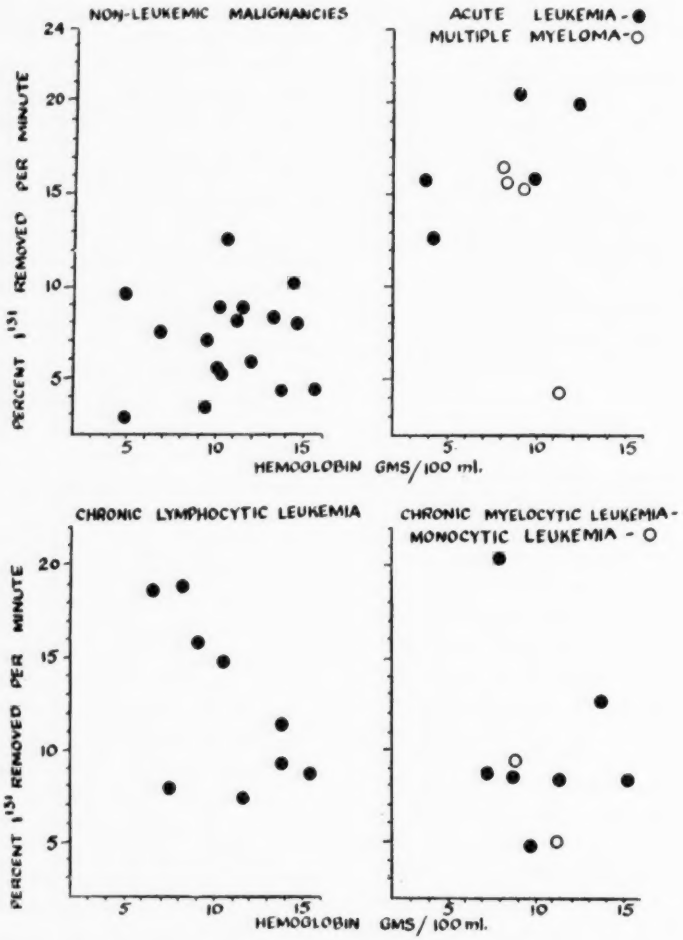

FIG. 2. PER CENT OF I^{131} CLEARED FROM THE MARROW PER MINUTE PLOTTED AGAINST THE HEMOGLOBIN CONCENTRATION

The relationship between marrow clearance and muscle clearance is presented as a ratio of marrow K to muscle K (Table I). These ratios are plotted against hemoglobin in Figure 3. The ratios of the non-leukemic, lymphoblastoma, and chronic myelocytic groups fall between 0.6 and 1.4 (Figure 3). In the group of chronic lymphocytic leukemia patients there was a scatter of the ratio values from 0.80 to 4.0, with five patients below and four above 1.4. In the two patients with acute leukemia the ratios were 4.1 and 3.42. The two patients with monocytic leukemia had ratios of 1.03 and 0.28, and in three cases of multiple myeloma, ratios of 1.67, 1.35, and 0.50 were obtained, respectively.

Although a wide distribution of Hb was present in all groups of patients, there appears to be no direct relationship between the marrow to muscle ratio and the hemoglobin concentration (Figure 3).

In a few patients it was possible to make additional clearance studies in order to ascertain the degree of reproducibility of the technique in the marrow. The data obtained in four patients are shown in Table III.

DISCUSSION

The validity of the radioisotope clearance method as a measure of effective local circulatory efficacy at the injected site was demonstrated theoretically and experimentally by Kety (3, 4). Stone and Miller (13) experimentally demonstrated that the radioactive sodium (Na^{24}) disappearing from the site of injection in the dog muscle can be quantitatively recovered in the venous blood draining the area of injection. Little or no radioactivity was recovered in the lymphatics draining the area. Subsequent studies by others (4–10) have supported the contention that locally injected radioisotopes can be used to determine the effective local blood flow in muscle and skin utilizing Na^{24} and I^{131}. Minor unexplained discrepancies have been detected between the clearance rates obtained with these two isotopes (8).

The extension of the Kety technique to a rigidly enclosed and trabeculated organ such as the bone marrow raises a question as to its validity as an index of regional blood flow in this organ. Certain experimental conditions which would be expected to affect the systemic blood flow were employed to demonstrate the validity

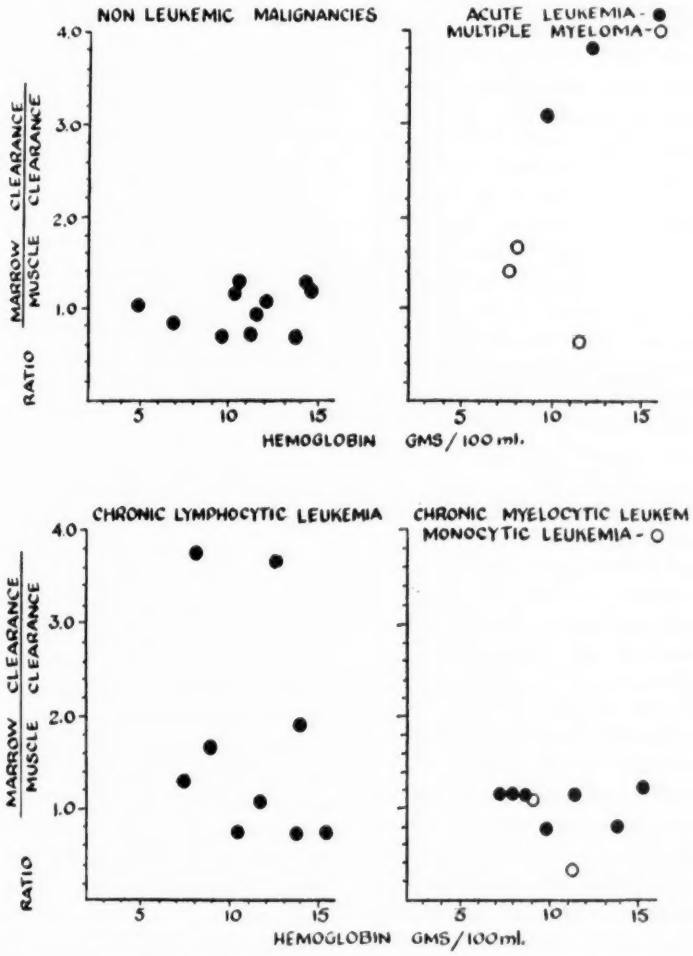

FIG. 3. RATIO OF MARROW CLEARANCE K TO MUSCLE CLEARANCE K PLOTTED AGAINST HEMOGLOBIN CONCENTRATION

TABLE III

Demonstrating reproducibility of bone marrow clearance constants

Patient	Date	Site	Marrow K
W. F.	9/6/51	Sternum	11.2
	9/6/51	Sternum	12.2
R. G.	9/13/51	Sternum	11.1
	9/14/51	Sternum	13.2
E. C.	7/23/51	Sternum	8.2
	7/23/51	Tibia	8.3
T. H.	7/23/51	Tibia	18.2
	9/ 6/51	Ilium	15.0

of this technique in bone marrow. Following the intravenous injection of hexamethonium bromide, a ganglionic blocking agent, both the marrow and the muscle clearance rates became very slow and appeared to be directly related to the pronounced fall in blood pressure which resulted (Figure 4). Conversely, the injection of Paredrine resulted in a distinct increase in the clearance rate from the marrow, presumably as a result of the sympathomimetic action of this drug. These preliminary experiments indicated that alterations in the systemic circulation are reflected in the I^{131} clearance rate from the bone marrow. It is therefore felt that the alterations in the bone marrow clearance rate produced by these pharmacologic agents further support the interpretation that the clearance technique is a measure of effective blood flow.

Of the many factors which could conceivably influence the clearance rate of I^{131} in the marrow, the volume of fluid injected is an important consideration. The sudden injection of a volume of fluid could possibly produce a local increase in tissue pressure which in turn might increase the initial rate of removal. With respect to bone marrow, the I^{131} was injected slowly, without pain, and a period of 1 to 2 minutes elapsed before counting was begun. This interval of time allowed decompression of the injected site to occur. In many of the cases, especially in those patients with acute leukemia and chronic lymphocytic leukemia, the injected I^{131} was literally aspirated into the marrow, indicating that no pressure developed from the injection. The role of the lymphatic drainage of I^{131} in the marrow is difficult to evaluate. However it is probably negligible, since no anatomic evidence for a lymphatic system in this organ has been presented (14).

Table III shows the degree of reproducibility of the marrow clearance values in the same patient on different occasions. Despite the technical difficulties encountered in making marrow clearance studies, it can be seen that a good agreement is obtained on repeated determinations.

The vascularity of the bone marrow is well recognized. It was demonstrated by Tocantins, O'Neill, and others that many substances, including blood, plasma, medications, and diagnostic agents, when injected into the marrow cavity rapidly appear in the systemic circulation (15, 16). Tocantins reported that fluids could be administered at a rate of from 0.4 cc. to 25 cc. per minute into the marrow, but this rate probably does not reflect the peak capacity for the removal of solutions injected into the marrow cavity because of changes in intramedullary pressure and physical disruption of the marrow vascular bed. These factors as well as the resistance to flow provided by the tubing, needle, and clotting of marrow blood probably exert a marked and variable effect on the rate of flow of substances introduced into the marrow. Jones (2) has estimated the rate of human hematopoietic marrow flow to be approximately 300 cc. per min. based on data obtained on rabbits in radioactive colloid uptake studies by the marrow.

The data obtained in the present study indicate that the clearance rates of I^{131} in such divergent types of vascular beds as bone marrow and resting muscle are essentially identical in patients with various malignancies, Hodgkin's disease and lymphosarcoma. These rates are considerably less than those reported by Wechsler, Sokoloff, and Kety (6) for human liver, where 34 to 48 per cent of the injected isotope was removed per minute. Striking increases were observed in the I^{131} clearance rates in the bone marrow in leukemic patients. The rapid clearance of radioiodide from the marrow in all the cases of acute leukemia, in half of those with chronic lymphatic leukemia, and in two of the three patients with multiple myeloma suggests that the marrow vascularity in these conditions is abnormal. In the patients with chronic myelocytic and monocytic leukemia studied, the clearance rates were similar to those found in the non-leukemic group of patients.

In an attempt to dissociate the systemic circula-

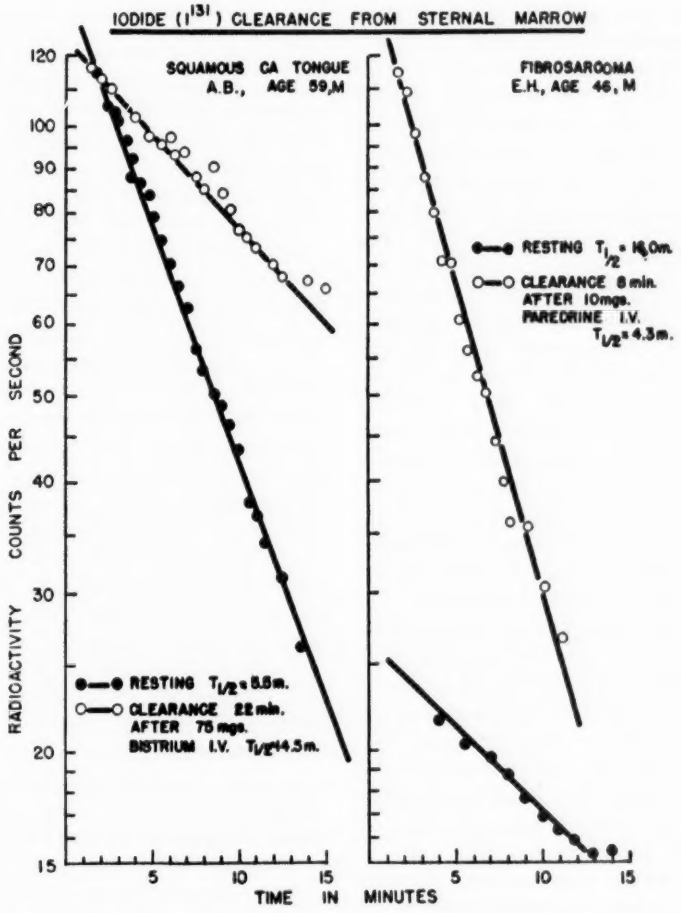

FIG. 4. ALTERATIONS PRODUCED IN I^{131} CLEARANCE FROM THE MARROW FOLLOWING THE INJECTION OF BISTRIUM AND PAREDRINE

tory influence on the local bone marrow flow, the data were expressed as a ratio of marrow K to muscle K. This ratio should serve to minimize the effect of any generalized increase in the cardiac output on the marrow clearance rate, such as might occur in exercise, emotional stress, anemia, etc. It will be noted that in all the patients studied, with the exception of those patients with acute leukemia, chronic lymphatic leukemia, and myeloma, the ratio of marrow K to muscle K falls below 1.4. It is felt that a clearly demonstrable specific local increase in marrow blood flow is present in those diseases with ratios above 1.4.

The demonstration of increased I^{131} clearance rates in the bone marrow in leukemia might be anticipated since these diseases have been considered to be neoplasms of the hematopoietic system (17). Many types of rapidly proliferating tumors have been demonstrated to elicit the formation of a vascular stroma (18, 19). The marked divergence between clearance rate found in those patients with acute and lymphocytic leukemia, and those with myelocytic and monocytic leukemia is of interest. The present findings indicate that fundamental differences may exist in the anatomy and physiology of the marrow vascular systems among the various human leukemias.

There is a lack of anatomical data on the vas-

cularity of both normal and pathological human bone marrow. A number of investigators (15, 20, 21) have attempted to visualize the sternal marrow vascular bed by injection of radiopaque substances, but these studies were unsatisfactory for the finer details of vascularity. No studies similar to those of Doan (22) in the pigeon were found for man. In observations made on histological sections of post-mortem bone marrow by Iwao (23) the marrow lesions in lymphocytic leukemia appeared to be associated with an increase in number of capillary sinuses, whereas no apparent change from the normal degree of vascularity was observed in the marrow in chronic myelocytic leukemia. Schleicher has also observed an increase in the degree of vascularity in the marrow associated with the patchy and generalized marrow lesions in chronic lymphocytic leukemia.[5] Such anatomic differences in the vascularity of the marrow may in part explain the findings obtained in the present study. Further detailed anatomic studies are needed with regard to these observations.

The bone marrow cytology and peripheral blood counts could not be related to the increased clearance rates, other than in establishing the diagnoses. While blast cells were present in the marrow in all cases of acute leukemia and appeared to be positively correlated with the rapid clearance rates found in these conditions, few or no lymphoblasts were observed in the chronic lymphocytic leukemia marrows, yet very rapid clearance rates were obtained. In one patient with multiple myeloma (V. W.) almost no cells of any type could be aspirated, yet the clearance rate was very rapid; 16 per cent per minute. These findings suggest that the nature of the vascular bed may be more important in determining the clearance rate rather than the presence or absence of abnormal cells in the marrow.

Although most of the patients studied had chronic anemia, no direct relationship was found between the clearance rate and hemoglobin level (Figures 2 and 3).

These preliminary observations give promise that the clearance rates of radioisotopes from the marrow may provide a means for the evaluation of systemic and local influences on the marrow circulation.

[5] Personal communication to authors.

SUMMARY AND CONCLUSIONS

The Kety radioisotope clearance technique with I^{131} has been applied to a study of bone marrow and muscle blood flow in 43 patients with various neoplastic diseases, lymphoblastomas, and leukemias. The marrow clearance rates were found to be approximately the same as resting muscle in all 17 of the non-leukemic patients studied.

In five cases of acute leukemia, marrow clearance rates were increased, being two to three times greater than the rate observed in the non-leukemic group, whereas, in five out of seven patients with chronic myelocytic leukemia, and in two cases of monocytic leukemia, the marrow clearance rates were within the range of the non-leukemic group of patients. In six out of ten patients with chronic lymphocytic leukemia, elevated clearance rates were obtained. Three out of four patients with multiple myeloma had increased marrow clearance rates. When marrow K to muscle K ratios were obtained in the non-leukemic, chronic myelocytic and monocytic leukemic patients, the values fell below 1.4. In two cases of acute leukemia and four out of ten chronic lymphocytic leukemics the ratio obtained was well above 1.4. The findings appear to demonstrate a selective increase in blood flow in the bone marrow in acute leukemia and in some cases of chronic lymphocytic leukemia and multiple myeloma, and strongly suggest that fundamental differences in the marrow vascular bed exist among the leukemic group of diseases.

Alterations were produced in the clearance rates in bone marrow following the intravenous injection of Paredrine and hexamethonium in two patients. The radioisotope clearance technique promises to be a useful tool for the investigation of the pathologic-physiology of the human bone marrow circulation.

ACKNOWLEDGMENT

We wish to express our appreciation to Dr. Jonah G. Li of the Division of Preventive Medicine, University of California School of Medicine, San Francisco for his cooperation in providing many of the patients for these studies.

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THE USE OF MODIFIED GLOBIN AS A PROTEIN SUPPLEMENT IN NORMAL INDIVIDUALS[1]

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In recent years there has been great interest in the use of substances which can be given intravenously to expand the volume of the blood. Perhaps for even a longer time there has been a search for protein substances which can be administered parenterally as food supplements. Plasma (1) and human serum albumin (2, 3, 4) have been used with partial success for both purposes. The latter, particularly, has been shown to be well utilized and easy to administer, but large quantities have proven to be time-consuming and expensive to prepare. With the present blood processing program there is inevitable wastage of human red blood cells and many people have felt that perhaps the protein moiety of such cells might be utilized for either of the above needs. Evidence exists that protein equilibrium can be maintained in animals with hemoglobin as the main source of protein (5).

Modified Globin[2] has been prepared from human red blood cells by partial hydrolysis. The globin has been characterized as having a uniform molecular weight of about 34,000. The final pH of the solution has been adjusted to 7.4. We have given this substance intravenously to patients as a supplement to a low protein diet. Metabolic studies were carried out in a manner similar to that used previously with human serum albumin (2). It was hoped that exactly comparable results could be obtained by the daily intravenous administration of the same amount (60 grams) of the test protein. Since the desired caloric intake could not be maintained in any patient when globin was given at such levels, this was not entirely realized. Nevertheless, certain fundamental differences between the metabolism of globin and albumin were apparent.

[1] This investigation was supported in part by a research grant from the National Heart Institute of the National Institutes of Health, Public Health Service.

[2] The globin used in these studies was prepared and generously supplied by Sharp & Dohme, Inc.

PATIENTS AND PROCEDURES

The clinical background of the four patients concerned in this study was as follows:

H. B., male, age 35, was admitted to the hospital for hysterical symptoms. He had lost about 20 lbs. in the year prior to this study. There was no evidence of organic disease.

G. V., male, age 25, had rheumatic heart disease with mitral stenosis and insufficiency, and aortic stenosis and insufficiency. This patient had been a subject on the metabolism ward twice before and had on one occasion received intravenous serum albumin. It was felt that comparative effects of albumin and globin in this patient would be of considerable interest.

E. C. was a 51 year old white male with probable peripheral neuritis. His admission to the hospital was precipitated by difficulty in walking. Although it is probable that alcoholism was responsible for his neurological difficulty, no evidence of liver disease was found.

P. B., female, age 26, had a severe psychoneurosis. The patient was poorly nourished, but had no organic disease.

Each patient was given a two-day rotating basic diet supplemented by calcium gluconate and KH_2PO_4. The menus for the two days varied but slightly and their composition was as close to identical as possible. According to repeated analyses of these diets the average daily intakes were as follows:

	N *Gm.*	P *Gm.*	Ca *Gm.*	Na *mEq.*	Cl *mEq.*	K *mEq.*	*Cal.*
H. B	4.03	1.04	.85	60	70	102	2502
G. V.	3.43	.99	.86	6	6	85	2230
E. C.	4.23	1.02	.91	63	70	103	2470
P. B.	3.77	.99	.84	47	51	78	2222

Analysis of the globin:

	N *Gm.*	P *Gm.*	Na *mEq.*	Cl *mEq.*	K *mEq.*
Lot No. 89049–4 (1 unit)	2.38	.006	21.5	16	.015
Lot No. 91213 (1 unit)	2.41	.004	16.9	8.5	.01

Additional sodium chloride was given in the control periods in amounts comparable to that which was contained in the globin solution. This was withdrawn during the time of intravenous globin administration.

With the exception of the last case, each patient was carried through an initial 12 day control study. In H. B. and G. V., during the first two experiments done, four units of globin were given each day in divided doses (each unit was dissolved in 400 cc. of 3½ per cent dextrose), the time of administration in each instance being about 1½ hours. In later experiments only two units of globin were given each day as supplements.

METHODS

The methods used were the same as those described previously in the work with human serum albumin given intravenously (2). In addition, globin levels in blood and urine were determined by the method of Kendall and Abell (6).

CLINICAL RESULTS

H. B.: Two hours following the first intravenous injection of two units of globin, the patient became nauseated. The second injection given four hours later was followed by a chill and temperature rise of 39.5° C. The patient again complained of nausea. From this time on the patient was given 50 mg. of pyribenzamine and two APC tablets one-half hour before each injection. He was asymptomatic on the second and third days. On the fourth day he again complained of nausea and vomited three times. The globin was, however, continued for a total of eight days. The vomiting became persistent on the seventh and eighth days, and the patient refused most of his diet at this time. After globin was stopped the patient continued to have nausea and emesis for four additional days. Following this, he was again able to tolerate his diet for a post injection control study of eight days.

G. V.: Shortly after the second injection on the first day of globin administration, the patient had a chill, felt "full," but continued to eat his diet. On the third day, after the morning injection, the patient complained of severe nausea, and refused to eat. The globin was stopped at this time, but nausea and vomiting occurred during the next two days.

After these two experiences it was decided to give only two units daily in the succeeding experiments. Another control period was, therefore, carried out on G. V. and then a series of injections of two units daily was given. (This has been called Experiment No. 2 on G. V. throughout the paper.) At this level the patient was able to tolerate globin for a ten-day period without undue symptoms. He did complain of nausea and headaches occasionally, but ate all his diet during this time and during the twelve-day control period after globin.

E. C.: This patient had no febrile response at any time. Following seven days of intravenous globin, two units each day, the patient stated that he was unable to eat his diet and the experiment had to be discontinued.

P. B.: This patient received two units of globin daily for seven days without incident. On the eighth day she complained of persistent nausea and refused to eat. She vomited frequently for the next two days and even after the globin was discontinued. Four days elapsed before the patient was able to eat her full diet.

LABORATORY RESULTS

Body weight

A small initial weight loss occurred in each patient which was regained as globin was continued. No striking effect on body weight was seen in any patient (see Table I).

Hematocrit

In the two patients who developed azotemia (H. B. and P. B.) a slight drop in hematocrit developed while the nitrogen retention was occurring. There were no changes in hematocrit which could be attributed to globin administration itself. This lack of change is in distinct contrast to that seen when albumin was administered, where repeated injections caused a progressive fall in the per cent of red cells.

Serum protein

There was no consistent change noted in any of the serum protein components with the exception of a slight increase of α_1 globulin found in all patients. In three patients there was no significant change in the albumin concentration, while in the fourth, H. B., the value fell .9 mg. per cent. This reduction in serum albumin concentration occurred during a period of azotemia which is discussed below.

Blood levels of globin

Only a few determinations of plasma globin levels were carried out. All samples were taken

TABLE I

Nitrogen balances

Patient	Period	Days	Body wt.	Nitrogen					Globin administered
				Intake	Urine	Stool	Emesis and refused food	Balance	
				Gm. per period	*Gm. per period*	*Gm. per period*	*Gm. per period*	*Gm. per period*	*Units per day*
			52.25						
H. B.	I	6	52.34	24.2	22.5	7.6	—	− 5.9	
	II	6	51.88	24.2	20.0	7.6	—	− 3.4	
	III	6	52.90	80.9	49.3	7.6	1.1	+22.9	4
	IV	3	53.25	31.1	11.0	2.8	3.6	+13.7	4
	V	6	50.81	24.2	49.4	5.6	3.6	−34.4	
	VI	6	50.34	24.4	38.1	5.6	—	−19.5	
			58.15						
G. V.	I	6	57.61	20.6	30.0	6.8	—	−16.2	
	II	6	57.87	20.6	19.4	6.8	—	− 5.6	
	III	3	56.78	34.3	27.8	3.1	—	+ 3.4	4
		3	56.08	10.3	16.6	3.1	3.3	−12.7	
	IV	4	57.79	13.7	16.4	4.2	—	− 6.9	
	V	6	56.41	49.4	38.3	5.8	—	+ 5.3	2
	VI	4	57.04	32.9	26.1	3.8	—	+ 3.0	2
	VII	6	56.10	20.6	25.3	5.8	—	− 7.5	
	VIII	4	56.21	13.7	15.7	4.3	—	− 6.3	
			58.72						
E. C.	I	6	58.78	25.4	22.5	10.6	—	− 7.7	
	II	6	58.95	25.4	21.4	9.5	—	− 5.5	
	III	6	57.47	54.3	41.1	8.2	—	+ 5.0	2
			54.4						
P. B.	I	6	54.33	22.6	15.1	7.4	—	+ 0.1	
	II	6	53.80	51.6	33.9	6.9	1.9	+ 8.9	2
	III	4	54.52	34.4	13.1	2.8	7.4	+11.1	2
	IV	6	51.79	22.6	29.2	4.1	11.2	−21.9	

at least 12 hours after any injection had been given. The values obtained were as follows:

1) H. B.—93 mg. per cent—after eight days of globin administration
2) G. V.—too low to measure after ten days of globin administration
3) P. B.—130 mg. per cent—after ten days of globin administration
4) E. C.—130 mg. per cent—after eight days of globin administration

These results suggest that even after repeated daily injections, there is little accumulation of globin in the serum.

Blood urea nitrogen

In two patients there was a progressive rise of blood urea nitrogen during the administration of globin, as seen in Table II. In H. B. it rose from 7 mg. per cent to 62 mg. per cent. During the twelve days of post injection study the level fell to 18 mg. per cent. Unfortunately, no kidney function studies were done on the patient prior to the injections; however, fifteen days after they were stopped para-amino-hippurate clearances were 463 and 408 ml. of plasma per minute and urea clearances during two consecutive hours were 39 per cent and 40 per cent of the normal average. One month later the urea clearances were 73 per cent and 75 per cent of normal. In P. B., the blood urea nitrogen rose from 4 mg. per cent to 31 mg. per cent. In this subject the urea clearance capacity was the same three weeks after globin had been discontinued as it had been prior to globin (72 per cent and 87 per cent before, and 74 per cent and 78 per cent after).

The rise of blood urea nitrogen as a point of evidence for lowered glomerular filtration is of some clinical importance, even though renal function apparently returned to normal in both instances. The possibility of dehydration from vomiting as a contributing cause has been considered but not thought responsible.

A careful accounting of the water exchange

showed that the intake, augmented by the intravenous fluids, was at all times greater than the output by a liberal margin, and the patients gained weight while azotemia and oliguria were developing.

Nitrogen balances

These are recorded in Table I and Figures 1 to 4. During the control periods on the basic low protein diets, three patients were in slightly negative nitrogen balance and one remained in balance.

In contrast to studies previously done with albumin (2) on the first day of globin administration the urinary nitrogen rose promptly (Figures 1–4). Each subject remained in positive nitrogen balance during the time of intravenous injections, but the amount retained was far less than was observed with comparable amounts of albumin. In one instance (P. B.), where dietary intake fell below caloric requirements, a negative nitrogen balance was seen during the last two days of globin administration (see period 3 in Figure 4).

The post injection control periods were of little value in H. B. and P. B., and in the first experiment on G. V., because of the inability of the patients to tolerate their diets. In the second experiment on G. V. conditions were satisfactory and one notes a nitrogen balance similar in all respects to that seen in the initial control periods (periods 7 and 8 in Figure 2).

TABLE II

*The levels of blood urea nitrogen before and during administration of globin**

Period	H. B.	G. V.	E. C.	P. B.
	mg. %	*mg. %*	*mg. %*	*mg. %*
0	10	14	8	8
I		7		4
II	7	5	4	[5]
III	[26]	[4]	[6]	[31]
IV	[62]	6		21–11
V	63–59–41	[8]		
VI	18	[10]		
VII		6		12

* The blocked-in numbers denote the periods during which globin was administered. Where single values are in the table they were obtained at the end of the designated periods. For length of periods see Table I. There was an interval of 14 days between the last two determinations on P. B.

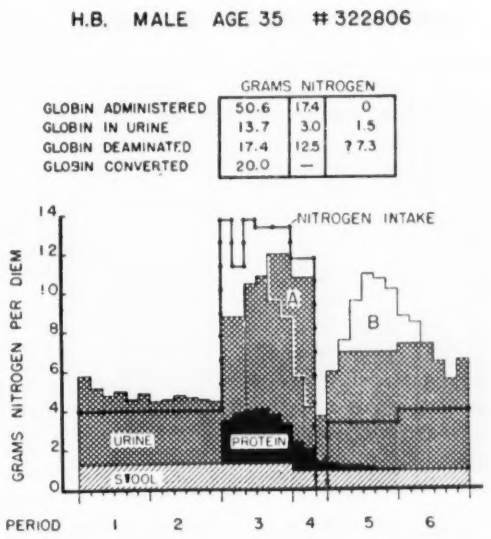

FIG. 1. NITROGEN BALANCE ON H. B. BEFORE, DURING, AND AFTER INTRAVENOUS ADMINISTRATION OF MODIFIED GLOBIN AS A SUPPLEMENT TO A LOW PROTEIN DIET

Area A represents protein deaminated and then temporarily retained. This was excreted as shown in area B. The cross-hatched area, therefore, shows the corrected urinary non-protein nitrogen and thus helps visualize the "true" metabolism of protein period by period.

Protein in urine

Quantitative values for urinary protein were determined in three ways:

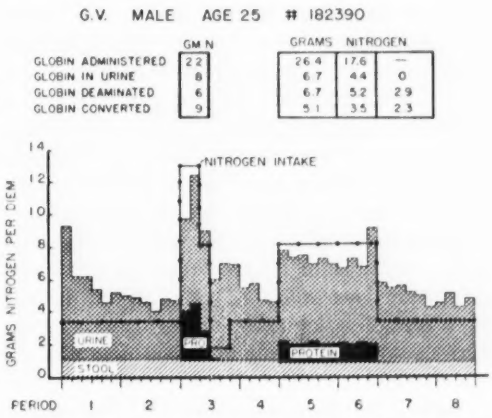
G.V. MALE AGE 25 # 182390
GM N
GRAMS NITROGEN
GLOBIN ADMINISTERED 2.2 26.4 17.6 —
GLOBIN IN URINE 8 6.7 4.4 0
GLOBIN DEAMINATED 6 6.7 5.2 2.9
GLOBIN CONVERTED 9 5.1 3.5 2.3
NITROGEN INTAKE
URINE
PROTEIN
STOOL
GRAMS NITROGEN PER DIEM
PERIOD 1 2 3 4 5 6 7 8

FIG. 2. NITROGEN BALANCE ON G. V. BEFORE, DURING, AND AFTER INTRAVENOUS ADMINISTRATION OF MODIFIED GLOBIN AS A SUPPLEMENT TO A LOW PROTEIN DIET

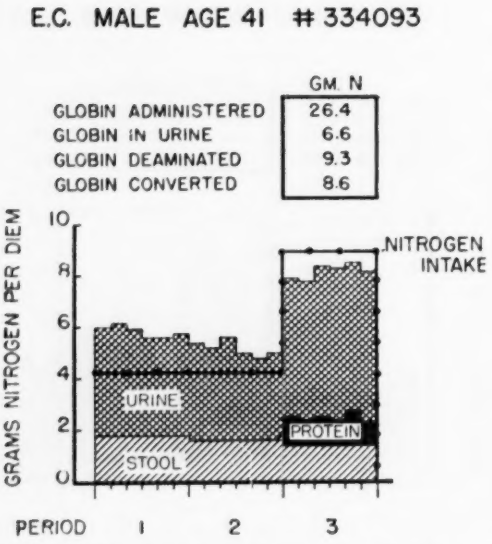

FIG. 3. NITROGEN BALANCE ON E. C. BEFORE AND DURING INTRAVENOUS ADMINISTRATION OF MODIFIED GLOBIN AS A SUPPLEMENT TO A LOW PROTEIN DIET

1) The biuret reaction (7).
2) Total nitrogen by the Kjeldahl Method minus the non-protein nitrogen in the urine.
3) Globin *per se* by the method of Kendall and Abell (6). This method depends on the observation that trichloracetic acid, buffered to a pH of 4.7 with sodium citrate, precipitates globin selectively.

By all three methods the results were quite similar. Figures derived from the biuret determinations only are given in Table III. Confirmatory evidence that this protein was globin and not albumin was obtained when precipitation with cationic detergents occurred at an isoelectric point quite different from that of albumin.[3]

It is of interest that the highest percentage of excretion occurred in G. V., who also excreted larger quantities of albumin than other subjects in the previous study (2). With H. B. and P. B. the decreasing excretion beginning after the sixth day occurred while the blood urea nitrogen was rising.

Aside from these variations the amount excreted in the urine each day was roughly proportional to the amount given. There was apparently no cumulative effect, the same amount having been excreted during the first day of administration as the last. There was no appreciable excretion of globin after the injections were stopped.

Phosphorus balances

Three of the four patients were in phosphorus equilibrium at the end of the control periods. As seen in Table II, retention of phosphorus occurred in all patients during the injections of globin. Although the daily values are not recorded in this paper, it should be stated that the drop of urinary phosphorus which occurred with globin was apparent on the first day. As will be discussed below, this may be interpreted to mean that conversion of this protein to body tissue begins promptly.

Urinary calcium excretion

It is worth noting that the urinary calcium decreased in all instances when globin was given, while for the most part the stool excretion was unaffected. The resultant positive balances, recorded in Table IV, were established. This ef-

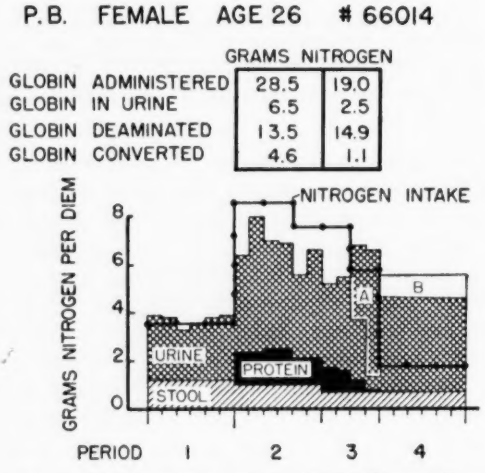

FIG. 4. NITROGEN BALANCE ON P. B. BEFORE, DURING, AND AFTER INTRAVENOUS ADMINISTRATION OF MODIFIED GLOBIN AS A SUPPLEMENT TO A LOW PROTEIN DIET

Explanation for areas A and B is the same as for Figure 1.

[3] These determinations were carried out through the courtesy of Dr. Eric L. Alling, Department of Radiation Biology, University of Rochester.

TABLE III

*Urinary excretion of globin**

Day	H. B.		G. V.				E. C.		P. B.	
	Amt. given	Amt. excreted	Amt. given Exp. No. 1	Amt. excreted Exp. No. 1	Amt. given Exp. No. 2	Amt. excreted Exp. No. 2	Amt. given	Amt. excreted	Amt. given	Amt. excreted
	Gm.	*Gm.*	*Gm.*	*Gm.*	*Gm.*	*Gm.*	*Gm.*	*Gm.*	*Gm.*	*Gm.*
1	64	13.8	64	18.5	32	7.7	32	7.4	32	6.6
2	48	10.3	64	21.8	32	6.9	32	6.2	32	6.7
3	64	16.9	32	10.9	32	6.7	32	7.2	32	7.6
4	64	17.4			32	7.8	32	6.6	32	7.4
5	64	15.6			32	6.9	32	7.9	32	6.9
6	64	12.6†			32	6.6	32	6.1	32	6.3
7	64	9.1			32	7.2			32	5.9†
8	64	6.9			32	6.7			32	5.8
9	0	2.7			32	7.1			32	3.5
10					32	6.6			32	.6

* The amounts of globin recorded here as having been given are according to the labels on the bottles, *i.e.*, 16 grams of globin per unit. The number of units varied from two to four daily. The intake values in all other tables and figures are taken from analyses performed on each lot.

† BUN started to rise.

fect has previously been observed when albumin was given. It was attributed to bone formation with the latter and may represent the same phenomenon in this instance.

Fate of the injected protein

An accounting of the injected globin was carried out in a manner similar to that used for serum albumin, *i.e.*,

1) Globin deaminated,
2) Globin converted to tissue protein,
3) Globin remaining unchanged in the body,
4) Globin excreted in the urine.

In calculating the deamination of globin it was assumed that as long as a patient consumed the same basal diet he deaminated protein other than globin at the same rate during the entire experiment. Whenever patients were unable to eat this diet or vomited, the nitrogen balance data were obviously not suitable for the calculations. It was also necessary to make adjustments for the observed changes in blood urea nitrogen (see Table II and Figures 1 and 4). Accordingly, the deaminated globin was considered to be represented by: the increased nitrogen excretion above control levels, minus nitrogen excreted as globin, plus the increase of urea nitrogen in the body. The urea was considered equally distributed throughout 60 per cent of the body weight.

The values as obtained on each patient are presented in Table V. They are stated as the amount of globin nitrogen deaminated and the percentage of the globin administered which this represents. It is important to emphasize that the deamination and excretion of globin began in each case during the first day of injection and continued at about the same levels until a critical reduction of caloric intake occurred (H. B., Period IV; P. B., Period III). As stated above, because of the dietary limitations, the values in the post injection control periods cannot be considered as valid criteria for the deamination of globin alone except in the case of G. V. In the post injection periods of the second experiment on this patient there was no evidence of increased excretion of nitrogen above the control level (Figure 2, periods 7 and 8). This indicates that

TABLE IV

Phosphorus, calcium and theoretical nitrogen balances expressed as deviations from the control periods

	Period	Days	Phosphorus balance	Calcium balance	Theoretical N balance
			Gm. per period	*Gm. per period*	*Gm. per period*
H. B.	III	6	+1.54	+.4	+20.
	IV	3	+ .02	−.24	+ 2.
G. V.					
No. 1	III	3	+ .73	+.30	+ 9.
No. 2	V	6	+ .35	+.06	+ 5.
	VI	4	+ .30	+.08	+ 4.
E. C.	III	6	+ .85	+.59	+ 9.
P. B.	II	6	+ .40	+.19	+ 5.
	III	4	+ .37	+.72	+ 1.

TABLE V

Deamination of globin

Patient	Period	Days	Globin given	Globin deaminated	
			Gm. N	*Gm. N*	*Per cent of amount given*
H. B.	III	6	50.6	17.4	34
	IV	3	17.4	12.5	72
G. V.					
No. 1	III	3	22.0	6.0	27
No. 2	V	6	26.4	6.7	25
	VI	4	17.6	5.2	30
E. C.	III	6	26.4	9.3	34
P. B.	II	6	28.5	13.5	47
	III	4	19.0	14.9	78

there was very little metabolism of the globin after the injections were stopped, a situation quite different from that when albumin was given.

The conversion of phosphorus-poor globin to tissue protein has been estimated by the retention of phosphorus associated with injections. The retention of phosphorus has, in each instance, been corrected for the amount which moved with the calcium for bone formation. The amounts of phosphorus and calcium retained have been recorded in Table IV as have the theoretical nitrogen balances (8) derived therefrom. The latter represent the estimated conversion of globin to tissue protein. It will be seen that in all patients this amount was considerable.

The summary data for the burning, excretion, and conversion of globin as percentages of the amounts given are recorded in Table VI. The following points must be emphasized:

1) A high percentage of the globin administered was deaminated and used for fuel only when caloric intake became inadequate (H. B., period IV; P. B., period III). At other times the conversion of this substance to body protein appeared to occur promptly and efficiently.
2) The amount of globin excreted as such in the urine was about one-quarter of the amount given except when renal insufficiency (as indicated by a rising blood urea nitrogen) became evident (H. B., period IV, P. B.; period III).
3) In nearly all periods the sum of the amount excreted in the urine, the amount deaminated, and the amount converted came to approximately 100 per cent. Thus, by exclusion, there was little evidence for storage of unchanged globin in the body at any time during the experiments. This finding correlates well with the low blood levels of globin and the failure of this substance to produce a sustained osmotic effect.

DISCUSSION

Studies with human serum albumin have indicated a slow degradation of this substance when given intravenously. When large amounts were given a half life of about five and one-half days was obtained (4). In studies in which the albumin was labeled biosynthetically with N^{15} or S^{35}, values for the half life time of 17 to 20 days were obtained (9, 10). The slow metabolism of this native protein is undoubtedly responsible for its prolonged osmotic effect. In contrast, when globin, the natural protein moiety of hemoglobin, was placed in the blood stream it was rapidly metabolized, probably within 24 hours. This fact may diminish the usefulness of this substance as a plasma extender. A transient increase of osmotic pressure may well have occurred with the globin administration, however, since our shortest experimental periods were 24 hours, and blood levels were determined 12 hours after administration.

Our data were derived mainly for the purpose of determining the nutritive value of this protein and, from this point of view, rapid metabolism would seem to be advantageous. Indirect evidence indicates that globin is readily converted to

TABLE VI

Summary of the fate of the globin administered intravenously expressed as per cent of the amount given

	Period	Days	Burned	Excreted	Converted	Total
			%	%	%	%
H. B.	III	6	34	27	40	101
	IV	3	72	17	11	100
G. V.						
No. 1	III	3	27	36	41	104
No. 2	V	6	25	25	19	69
	VI	4	30	25	20	75
E. C.	III	6	34	25	33	92
P. B.	II	6	47	23	17	87
	III	4	78	13	6	97

tissue protein, but as always, one must have an adequate caloric intake to accomplish this. The invariable production of the symptoms of nausea and vomiting, therefore, becomes an important factor in the use of globin as a nutritive supplement. The gastrointestinal difficulty was apparent in each patient after about 12 units had been injected. The cause remains undetermined. Amino acid injections are prone to cause symptoms of nausea and vomiting, although some difference of opinion still exists as to the determinant factor in this reaction (1). The globin was analyzed for non-protein nitrogen on several occasions and less than one-tenth of the material was not in the form of protein. It seems unlikely therefore, that amino acids were responsible for the reactions. As pure speculation, from the pattern of the symptomatology, one might suggest that some product of the hydrolysis, probably not an amino acid, accumulates during repeated injections and eventually produces the symptoms of gastro-intestinal distress.

SUMMARY

The evidence we have accumulated indicates that:

1) In normal individuals about 25 per cent of injected globin was excreted promptly as such in the urine.
2) There was insignificant accumulation of globin either in the plasma or the extracellular fluid. Metabolism occurred promptly.
3) Amounts comparable to albumin were converted to tissue protein, if the caloric intake was adequate.

Since the preparation of this manuscript a paper has been published of studies on the metabolism of globin, as determined by radioactive labeling. Evidence for rapid metabolism of globin was also found by this method. (Berson, S. A., Yalow, R. S., Post, J., Wisham, L. H., Newerly, K. N., Villazan, M. J., and Vazquez, O. N., Distribution and fate of intravenously administered modified human globin and its effect on blood volume. Studies utilizing I^{131} tagged globin. J. Clin. Invest., 1953, **32**, 22.)

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THE REGULATION OF RENAL BICARBONATE REABSORPTION BY PLASMA CARBON DIOXIDE TENSION [1]

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INTRODUCTION

Pitts, Lotspeich, Ayer, and Schiess (1, 2) have shown that in acute metabolic acidosis or alkalosis the normal kidney handles bicarbonate as if there were a maximal rate of tubular reabsorption which is a direct linear function of the glomerular filtration rate (approximately 2.5 to 2.8 mEq. HCO_3 per 100 cc. GFR, in dog and man). At plasma bicarbonate concentrations significantly below 25 mEq. per L., bicarbonate is completely reabsorbed and the urine is acid. When plasma bicarbonate concentration is elevated above this threshold value all the filtered bicarbonate in excess of the limiting rate is excreted in the urine.

In respiratory disturbances of acid-base balance, however, the bicarbonate threshold must be changed considerably, because in respiratory alkalosis the urine is alkaline despite low plasma bicarbonate concentrations (3–5) and in respiratory acidosis it is acid when plasma bicarbonate is high (6). The mechanisms initiating these changes in bicarbonate transport have not yet been elucidated, although from *a priori* considerations one would suspect that they must operate through a change in either carbon dioxide tension or extracellular fluid pH. The purpose of this report is to present observations demonstrating that respiratory regulation of bicarbonate reabsorption is effected through changes in carbon dioxide tension and is independent of extracellular fluid pH.

METHODS AND MATERIALS

Eleven experiments were performed on ten female mongrel dogs weighing 14 to 21 Kg. Light general anesthesia was maintained by the intravenous administration of sodium pentobarbital (12.5 mg. per Kg. as a 2.5 per cent solution). A Magill endotracheal tube fitted with an inflatable balloon was introduced into the trachea and connected in series with a Mine Safety "Pneophore." Respiratory movements were inhibited by the administration of d-tubocurarine chloride (0.7 mg. per Kg.) so that respiratory exchange could be controlled by the intermittent positive pressure device.

In order to assure complete oxygen saturation of arterial blood at all times the animals breathed 95 per cent or 100 per cent oxygen mixtures throughout each experiment. On each of the several occasions when it was checked, saturation of arterial hemoglobin with oxygen was complete despite the wide variations in pCO_2. The desired plasma bicarbonate concentrations were achieved by rapid intravenous injection of sodium bicarbonate and maintained by continuous infusion of an approximately isotonic solution of an appropriate mixture of sodium bicarbonate and sodium chloride given at a steady rate of 9.5 cc. per min. through a Bowman infusion pump. Glomerular filtration rate was measured by the inulin clearance technique. Frequent blood samples were withdrawn anaerobically into heparinized syringes through a plastic arterial cannula. Urine was collected under neutral mineral oil through an inlying bladder catheter and the bladder was emptied by manual compression at the end of each period. Urine collection periods were usually 10 to 12 minutes long. They were always begun at least 40 minutes after any change in plasma pH or bicarbonate had been initiated, and at least 20 minutes after plasma pH and bicarbonate had become essentially constant.

Urine and plasma were analyzed for inulin (7) and for total CO_2 content (8). Blood and urine pH were determined anaerobically at room temperature in a syringe-type Cambridge pH meter and corrected to 37° C., using an adjustment of .01 pH unit per degree. Carbon dioxide tension in blood and urine was calculated from the Henderson-Hasselbalch equation, using a value of 6.1 for the pK in both instances and a value for α in blood of .0301 and in urine of .0309. Bicarbonate concentrations in plasma and urine were calculated as the difference between the total CO_2 content and the dissolved carbonic acid. Filtered bicarbonate was taken as the product of the filtration rate and the plasma bicarbonate concentration.

Three types of experiments were carried out. In the first type, while the animals breathed pure oxygen, carbon dioxide tension was varied by changing pulmonary ventilation. This produced large changes in pH, but the infu-

[1] Supported in part by grants from the Medical Research and Development Board, Department of the Army, and from the National Heart Institute of the National Institutes of Health, U. S. Public Health Service.

sion of sodium bicarbonate assured plasma bicarbonate concentrations high enough to maintain an alkaline reaction (*i.e.*, plasma pH always greater than 7.45). To control the possible role of neural or humoral influences arising from changes in respiratory mechanics, similar variations in pCO_2 were produced with no change in ventilation by the substitution of a 5 per cent carbon dioxide, 95 per cent oxygen mixture for the pure oxygen. In a third group of experiments, pCO_2 and plasma bicarbonate were changed simultaneously so that a constant, alkaline plasma pH was maintained during all the observation periods. This eliminated the possible influence on renal bicarbonate transport of a change in extracellular pH. Urine bicarbonate excretion in every experiment was always sufficiently high to indicate that tubular reabsorptive capacity had been exceeded.

RESULTS

In all experiments an increase in plasma pCO_2, with or without change in extracellular pH, and with or without a change in ventilation, significantly increased the rate of bicarbonate reabsorption, and a decrease in plasma pCO_2 decreased bicarbonate reabsorption. The changes in pCO_2 produced no consistent or significant alterations in glomerular filtration rate, although in a few experiments the filtration rate tended to decline slightly as the experiment proceeded.

Table I gives the protocol of an experiment typical of seven, in which pCO_2 was varied by changing ventilation. The data are illustrated in Figure 1. It is seen that with each change in pCO_2 there was an associated change in bicarbonate reabsorption, expressed either in absolute terms (Table I) or corrected to 100 cc. GFR. The bicarbonate concentration in the reabsorbate varied with the pCO_2 in a similar fashion (Table I and Figure 1). It should be noted that during the last three urine periods the GFR was slightly reduced, but the acute changes in reabsorbate concentration were independent of this phenomenon. The plasma pH never was lower than 7.46. The changes in plasma bicarbonate level apparently occurred as a result of the renal response to the altered pCO_2. In this experiment, as in all others in which the sustaining infusion was not changed, there were no consistent changes in urine flow.

In two experiments in which plasma pCO_2 was varied, without changing ventilation, by switching to a 5 per cent CO_2 and 95 per cent oxygen mixture, the results were entirely comparable to those already described.

Table II and Figure 2 present the data from one of two other experiments in which the plasma pCO_2 was varied without alteration in plasma pH by simultaneously changing ventilation and the plasma bicarbonate level. Plasma pH was never lower than 7.53. It can be seen that the effect on bicarbonate reabsorption was similar to that illustrated in Table I and Figure 1. In this experiment, the rise in plasma bicarbonate levels and the increased urine flow during the third and fourth periods were probably due mainly to the large intravenous load of sodium bicarbonate. In the two experiments of this type, prolonged periods of equilibration were required between succesive observation periods in order to adjust plasma bicarbonate and pCO_2 to the desired levels.

In Figure 3 are plotted all the data taken from fifty-four observations in the eleven experiments

TABLE I

Effect of changing CO_2 tension on bicarbonate reabsorption during metabolic alkalosis

Dog Jackie: Weight 14 Kg. Loaded with 180 mEq. $NaHCO_3$ at 0 time and sustaining infusion containing 100 mEq./L. $NaHCO_3$ and 50 mEq./L. NaCl started at 9.5 cc./min.

Total elapsed time,	Plasma			Glom. filt. rate	Urine flow	$[HCO_3]$		
	pH	pCO_2	$[HCO_3]$			Excreted	Reabsorbed	Reabsorbate concentration
min.		*mm. Hg*	*mEq./L.*	*cc./min.*	*cc./min.*	*mEq./min.*	*mEq./min.*	*mEq./L.*
103–113	7.83	30	48.4	40	11.3	1.17	.760	26.6
113–123	7.84	29	47.5	44	13.9	1.35	.720	24.3
126 Ventilation reduced								
202–212	7.46	83	55.8	49	12.9	1.33	1.40	38.8
212–222	7.48	78	55.3	41	10.9	1.12	1.16	38.2
223 Ventilation increased								
285–295	7.81	36	53.8	33	11.3	1.10	.670	31.2
295–305	7.84	34	54.1	30	9.8	1.02	.620	30.2
307 Ventilation reduced								
346–356	7.53	73	59.4	30	8.3	.76	1.05	47.5

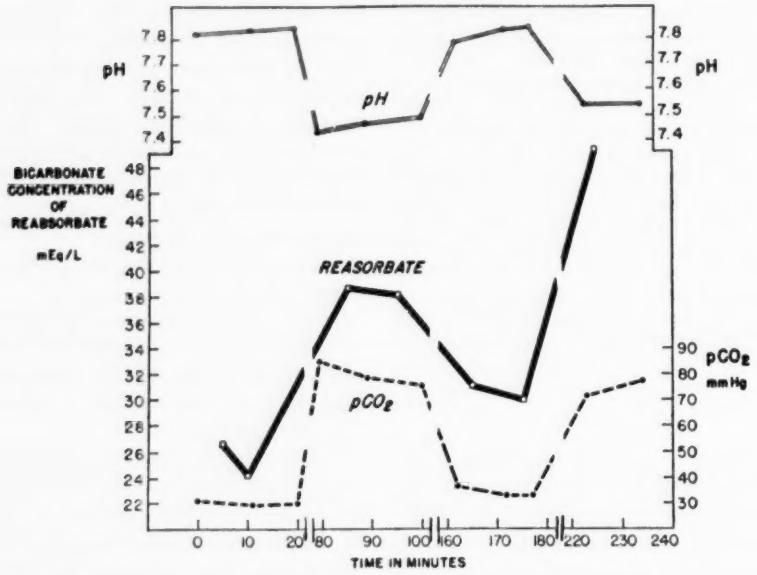

FIG. 1. THE EFFECT OF CHANGING pCO_2 ON BICARBONATE REABSORPTION IN THE PRESENCE OF METABOLIC ALKALOSIS

In this experiment pCO_2 was increased by hypoventilation. The breaks in the ordinate scale, in this figure and in Figure 2, represent the passage of time during equilibration.

reported here. The graph demonstrates the relationship between pCO_2 and the bicarbonate concentration of the reabsorbate, when pCO_2 is varied between 14 and 91 mm. Hg in the presence of a metabolic alkalosis (pH 7.45 to 7.93). It can be seen that there is a good, direct linear correlation between these two parameters ($r = .80$). The correlation between pCO_2 and bicarbonate threshold expressed as mEq. HCO_3 reabsorbed per 100 cc. GFR × 10 was approximately the same ($r = .91$); the regression equation for pCO_2 on this latter parameter was $y = .28x + 12.3$ and the equation for pCO_2 on the concentration of the reabsorbate was $y = .38x + 13.7$.

DISCUSSION

It seems clear from these observations that, contrary to previous opinion (5, 6), renal bicarbonate

TABLE II

Effect of changing CO_2 tension on bicarbonate reabsorption in presence of constant plasma pH

Dog Josie: Weight 16 Kg. Loaded with 72 mEq. $NaHCO_3$ at 0 time and sustaining infusion containing 45 mEq./L. $NaHCO_3$ and 105 mEq./L. NaCl started at 9.5 cc./min.

Total elapsed time,	Plasma			Glom. filt. rate	Urine flow	[HCO_3]		
	pH	pCO_2	[HCO_3]			Excreted	Reabsorbed	Reabsorbate concentration
min.		*mm. Hg*	*mEq./L.*	*cc./min.*	*cc./min.*	*mEq./min.*	*mEq./min.*	*mEq./L.*
78–90	7.54	34	27.9	72	5.8	.402	1.62	24.3
90–102	7.56	33	28.1	72	7.1	.418	1.61	24.6
108 Loaded with 285 mEq. $NaHCO_3$ and sustaining infusion changed to 180 mEq./L. $NaHCO_3$. Ventilation reduced.								
328–339	7.57	78	67.7	62	14.5	2.06	2.11	44.8
339–350	7.57	75	67.0	59	13.9	2.04	1.92	42.5
353 Sustaining infusion changed to 150 mEq./L. NaCl. Ventilation increased.								
580–590	7.53	39	30.8	64	6.1	.609	1.34	23.4
590–600	7.54	36	29.9	63	6.3	.574	1.33	23.3

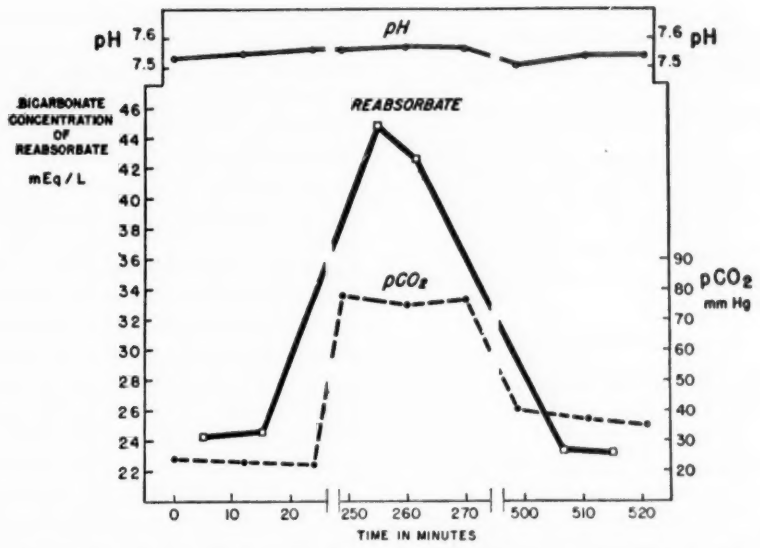

FIG. 2. THE EFFECT OF CHANGING pCO_2 ON BICARBONATE REABSORPTION IN THE PRESENCE OF A CONSTANT, ALKALINE PLASMA pH

The pH was kept constant by adjustment of plasma bicarbonate level with each change in pCO_2.

reabsorption is directly related to the carbon dioxide tension of body fluids and is independent of extracellular fluid pH. The data imply that the increased renal threshold for bicarbonate in respiratory acidosis is the result of the increased carbon dioxide tension and does not depend on either the existence of an extracellular acidosis or on any change in blood pH. The data also imply that alkalinuria in respiratory alkalosis is the result of the lowered plasma carbon dioxide tension and not of the elevated plasma pH.

According to these findings, compensatory hypoventilation in chronic metabolic alkalosis, with an attendant rise in pCO_2 should increase renal bicarbonate reabsorption. That bicarbonate reabsorption is, in fact, increased in severe compensated, or partially compensated, metabolic alkalosis is not generally appreciated, although calculation shows that such must usually be the case, even when the urine is alkaline. In Table III are calculated the urine bicarbonate concentrations which would obtain in an hypothetical alkalotic patient with a plasma bicarbonate concentration of 40 mEq. per L. and a "normal" rate of bicarbonate reabsorption, *e.g.*, 2.8 mEq. per 100 cc. GFR (2). The calculation is made for two different values of GFR and three different daily urine volumes. It can be seen that only at very high urine flows and low filtration rates could the urine bicarbonate concentration be less than 200 mEq. per L. At the more usual urine flows and glomerular filtra-

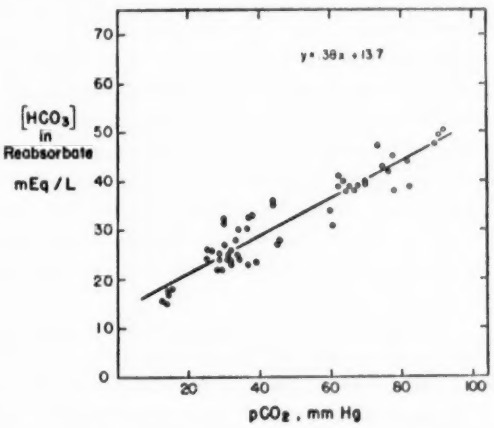

FIG. 3. THE RELATION OF REABSORBATE BICARBONATE CONCENTRATION TO pCO_2 FOR ALL EXPERIMENTS

The plasma pH was never less than 7.45.

TABLE III

Urine bicarbonate concentrations which would be required in a patient with a plasma bicarbonate of 40 mEq./L. and a "normal" rate of bicarbonate reabsorption (2.8 mEq./100 cc. GFR)

Glom. filt. rate *cc./min.*	Urine volume *Liter/day*	Estimated urine HCO_3 concentration *mEq./L.*
100	1	1730
	2	865
	5	346
50	1	865
	2	432
	5	173

tion rates, "normal" rates of bicarbonate reabsorption would result in urine concentrations much higher than the highest values observed during extreme bicarbonate loading in man and dog (1–3). Furthermore, the calculated daily bicarbonate excretion would be large enough to remove rapidly excess alkali from the body in almost all conceivable clinical situations.

It is, therefore, probable that in most patients with severe chronic alkalosis renal reabsorption of bicarbonate is increased even when the urine is alkaline. The difference between these patients with alkaline urines, and those with so-called "paradoxical aciduria," is probably only a matter of degree since it would appear that neither group is excreting bicarbonate "normally." The rise in bicarbonate reabsorption resulting from hypercapnia is clearly useful to the organism defending against respiratory acidosis, but it appears to be inappropriate in metabolic alkalosis where it restricts the renal elimination of alkali and tends to perpetuate the elevation of the plasma bicarbonate concentration. It is probably fortunate for the organism that the respiratory response to acute metabolic alkalosis is often slow (9) and incomplete (2, 3) because the kidney is thereby permitted to excrete the excess alkali at a maximal rate and restore plasma bicarbonate, as well as plasma pH, to normal values.

Although the evidence presented here indicates that any compensatory retention of carbon dioxide will tend to elevate the renal bicarbonate threshold in alkalosis, other factors may be operating as well. Sodium depletion (4, 6, 10) and potassium depletion (11) have been implicated as causes of "paradoxical aciduria," and recently it has been shown that acute experimental reductions in glomerular filtration rate will also raise the renal bicarbonate threshold (12). One or more of these latter factors may act together with an elevated pCO_2 to decrease bicarbonate excretion in any particular case of chronic alkalosis. For example, in the case of gastric alkalosis with "paradoxical aciduria" studied by Kennedy, Winkley, and Dunning (13) the plasma pCO_2 was about 60 mm. Hg, but there was also severe potassium depletion as well as a moderate reduction in glomerular filtration rate. The present experiments were also occasionally complicated by small variations in GFR (Table I), and all animals developed some degree of hypokaliemia as the result of prolonged loading with sodium bicarbonate solutions (14). The scatter of points in Figure 3, as well as the failure of pCO_2 to correlate strictly with bicarbonate reabsorption in some individual dogs (Figure 1), may be attributable in part to one or both of these factors.

Despite the probability that reabsorbate concentration is also affected by changes in urine flow (15), the correlation between pCO_2 and reabsorbate concentration was chosen for illustration because it best describes how changes in pCO_2 would affect renal regulation of plasma concentration under normal circumstances. It should be noted, however, that bicarbonate reabsorption calculated as mEq. per 100 cc. GFR correlates with pCO_2 in essentially the same way. This indicates that despite the variations in urine flow, the use of reabsorbate concentration is valid for the purposes of discussion in this study.

Examination of Figure 3 suggests that the relation betwen pCO_2 and reabsorbate concentration may be the manifestation of a renal mechanism tending to stabilize extracellular fluid pH. If one assumes a constant, normal pH of 7.35 for the dog, it is possible to calculate from the Henderson-Hasselbalch equation a theoretical relationship between pCO_2 and reabsorbate concentration. This line ($y = .53x$) has a slope very similar to that of the observed regression line ($m = .38$), although the latter defines a small variation in reabsorbate pH (pH 7.48 at $pCO_2 = 40$ mm. Hg; pH 7.36 at $pCO_2 = 80$ mm. Hg). It is impossible from the data at hand to determine how much of this variation may be due to experimental error or to complicating factors such as potassium depletion.

However, even if the observed line is an accurate description of the renal response, the relatively narrow limits of pH variation indicate that the mechanism would defend extracellular pH with considerable efficiency over a wide range of pCO_2. The existence of such a mechanism is further suggested by the clinical observation (16) that extracellular pH is often within normal limits in patients with primary CO_2 excess.

These experiments provide no clue to the means by which the pCO_2 exerts its effect on bicarbonate reabsorption. One possibility is that hypercapnia increases acidity within renal tubular cells, producing a condition comparable to that resulting from potassium deficiency (11, 14). Another possibility is that the high pCO_2 increases carbonic anhydrase activity (17) and thereby accelerates bicarbonate transport. Experiments to be reported from this laboratory (18) indicate that renal carbonic anhydrase activity is probably increased when pCO_2 is high and that under these circumstances the increased enzyme activity may be responsible for the augmented transport of bicarbonate.

SUMMARY AND CONCLUSIONS

There is a direct, approximately linear relationship between plasma carbon dioxide tension and renal bicarbonate reabsorption in the anesthetized dog which can be demonstrated in the presence of extracellular alkalosis and does not require any change in pulmonary ventilation or in extracellular pH. It is suggested that renal adjustment of bicarbonate reabsorption is related to carbon dioxide tension in a manner which tends to preserve the neutrality of the reabsorbate and the extracellular fluid. The data indicate that changes in pCO_2 are primarily responsible for the increased bicarbonate threshold in respiratory acidosis and the reduced threshold in respiratory alkalosis. Respiratory compensation of metabolic alkalosis increases the bicarbonate threshold and thus tends to perpetuate the elevation of extracellular bicarbonate concentration.

The mechanism of action of pCO_2 on bicarbonate transport is not revealed by these experiments, but it is suggested that changes in acidity or carbonic anhydrase activity within renal tubular cells may be responsible.

ACKNOWLEDGMENTS

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Addendum. Independent observations by P. Brazeau and A. Gilman (Federation Proc., 1953, **12**, 19) and by P. J. Dorman and W. J. Sullivan (Personal communication) are in substantial agreement with the findings reported here.

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A CLINICAL AND EXPERIMENTAL STUDY OF THE ERYTHROCYTE ULTRASTRUCTURE MEMBRANE WITH THE ELECTRON MICROSCOPE [1]

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Increasing interest is being focused on the various modifications and environmental influences which may render red blood cells progeric and susceptible to one of the destructive mechanisms. In addition to the study of the intact erythrocyte and its environment, investigations of the two primary components, *i.e.*, the hemoglobin and the ultrastructure, have been particularly fruitful in the last fifteen years. The surface ultrastructure has been fairly well defined chemically and physically (1). The more recent work on identification of genetically different chemical types of human hemoglobin has thrown new light on the hemoglobin-ultrastructure relationship, but has also revealed additional problems (2–4).

Most of the previous observation on erythrocyte ultrastructure by means of the electron microscope have been confined to a single type of red blood cell (5–8). It was our intention to make a broader study of the ultrastructure with the hope of obtaining a better understanding of erythrocyte destruction. The following types of red blood cells were included: Normal erythrocytes and erythrocytes from patients with various hematologic diseases; erythrocytes modified by sensitization, enzyme treatment (trypsin), roentgen radiation, *in vitro* and *in vivo*, storage, incubation, treatment with various chemicals, and varying pH.

MATERIALS AND METHODS

I. *Preparation*

A. General. Unless otherwise specifically noted, the following procedures were used:

1. *Collection and washing of erythrocytes:* Four ml. of intravenous blood were collected in 1 ml. ACD solution (Baxter). After removal of plasma by centrifugation, blood cells were washed three times with saline.[2]

2. *Preparation of membranes:* Freshly collected, washed erythrocytes were hemolyzed by addition of 100 to 200 volumes of distilled water (pH 6.5). The suspension was allowed to stand at room temperature for 10 minutes. Membranes were separated by centrifugation and washed six times with distilled water.

3. *Preparation of screens for electron microscope:* One drop of a thin suspension of the membranes or erythrocytes was placed on a Formvar [3] membrane screen and dried at room temperature. *Fixatives were not used.*

B. Preparation of membranes from one particular case of idiopathic acquired hemolytic anemia (W. T.).

Sixty per cent of the red cell population in this case (W. T.) consisted of spherocytes. In order to obtain a higher yield of spherocytic membranes, the freshly collected sample was allowed to stand for 10 minutes at room temperature. Spontaneous hemolysis of a large portion of the red cells occurred. The hemolyzed cells were assumed to have been spherocytes. The remaining intact erythrocytes were then removed by slow centrifugation (1,000 rpm. for 5 minutes). The major portion of the membranes from hemolyzed erythrocytes were thus retained in the supernatant plasma. Resuspension in saline of the intact erythrocytes, followed by further slow centrifugation, yielded additional spherocytic membranes. The membranes were then washed six times with distilled water.

C. Modified erythrocytes and membranes.

1. *Chemical treatment:* The following chemicals were used in treating erythrocytes and membranes: osmic acid 1 per cent, formalin 1 per cent and 10 per cent; 1 per cent solutions of copper sulfate, lead nitrate, ferric sulfate, mercuric chloride and silver nitrate.

(a) Two-tenths ml. of normal packed washed RBC were incubated with 5 ml. of the chemical solution for 10 minutes at room temperature.

(b) Five ml. of normal membrane suspension were incubated with 5 ml. of chemical solution at room temperature. Membranes were then washed three times with distilled water.

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[2] All saline is physiologic in this study.

[3] A resin manufactured by the Shawinigan Products Corp., New York, N. Y.

2. *Effect of pH:* Normal, washed erythrocytes were hemolyzed as in section *A* above, except that altered pH values of the distilled water, as checked with a Beckman pH meter, were used.

3. *Trypsin treatment:*

(a) Treatment of the intact erythrocyte: One volume of normal, washed red cells was incubated with one and one-half volumes of trypsin [4] solution at 37° C. for 30 minutes, then washed three times with saline. Concentrations of trypsin ranged from 0.1 to 4.0 per cent.

(b) Treatment of normal membranes: One drop of the desired trypsin solution was placed on the erythrocyte membrane previously dried on the Formvar screen. Following incubation at 37° C. for half an hour, the trypsin solution was decanted off, and the screen allowed to dry at room temperature.

4. *Sensitization of erythrocytes:* Group A and B human erythrocytes were sensitized with anti A and anti B sera (Lederle) in various dilutions. Two-tenths ml. of washed, packed cells and 1 ml. of the serum dilution were incubated for 30 minutes at 37° C. Following sensitization, the cells were washed three times, and the membranes prepared as outlined under section *A* above.

5. *Irradiation of erythrocytes:*

(a) *In vivo.* Domestic rabbits were given 850 to 900 r total body irradiation. Blood specimens were taken by cardiac puncture at 24 hours postirradiation, and every 48 hours thereafter as long as the animal survived.

(b) *In vitro.* Normal erythrocytes were washed five times with saline. One ml. of a 1:1 cell-saline suspension was placed in a paraffin coated tube (1 cm. diameter) and irradiated in doses ranging from 24,000 r to 96,000 r (140 KV, no filter, 8 cm., 7 mA, 2087 r per min.). After irradiation the erythrocytes were hemolyzed immediately, and the membranes prepared in the usual manner.

6. *Stored erythrocytes:* Bank bloods, collected in ACD solution, and stored at 4° C. were examined weekly for as long as 20 weeks.

7. *Influence of temperature on normal erythrocytes and membranes:* The erythrocytes or membranes were treated in the following manner:

(a) Sterile, defibrinated blood samples were incubated at 37° C. for ½ to 24 hours.

(b) Saline suspensions of normal, washed erythrocytes were heated for 30 minutes at 56° C.

(c) Normal, washed red cells were hemolyzed in distilled water at 56° C. for 10 minutes.

(d) Normal membranes suspended in distilled water were heated at 56° C. for 30 minutes.

(e) Normal membranes were stored in distilled water (pH 6.5) at 4° C. for 21 days.

II. *Observations*

Fifty to one hundred membranes or red cells were examined in each specimen. In addition to the fluorescent screen study, the observations reported here were also based on the detailed study of approximately 2,400 electron microphotographs.

Observations and photographs were made with the electron microscope type EMU, RCA. In the instances in which chromium and gold shadow casting were used, the technic was that described by Wyckoff (9). Details are given in the captions.

RESULTS

I. *Normal erythrocyte membranes*

Forty-three samples of erythrocytes from thirty healthy individuals were examined. Only minimal differences in the membranes of the red cell spectrum could be detected; thus, for example, it was not possible to distinguish the young from the aged circulating cell.

The normal erythrocyte membrane appeared as a thin-walled double disc, comparable to the flattening of a round balloon. The diameter was slightly larger than that of the intact red cell. The surface presented a finely granular appearance (Figure 1).[5] The great majority of the membranes showed no holes; however, rarely one (Figure 2) or more holes (Figure 3) were evident. Three of the 43 specimens showed fold formation in 1 to 3 per cent of the membrane population. Fold formation is discussed more fully in a following section. Unhemolyzed erythrocytes often showed a less dense center in electron micrographs, evidence of less hemoglobin in this area. In partially hemolyzed erythrocytes, a density of hemoglobin was seen at the periphery (Figure 4).

[4] Difco trypsin, 1:250 activity.

[5] Recent reports by Bessis, Ponder and their associates (20, 21, 22) that have come to our attention since the preparation of this manuscript have described a most interesting structure that they observed in erythrocyte ultrastructure membranes with the electron micrograph technics. This filamentous structure apparently originates in small craters of the ultrastructure and when observed on the periphery gives a whiskered appearance. They called these hematexodies. These were not observed in our preparations; this was probably due to our more intense washing of the membranes.

PLATE I

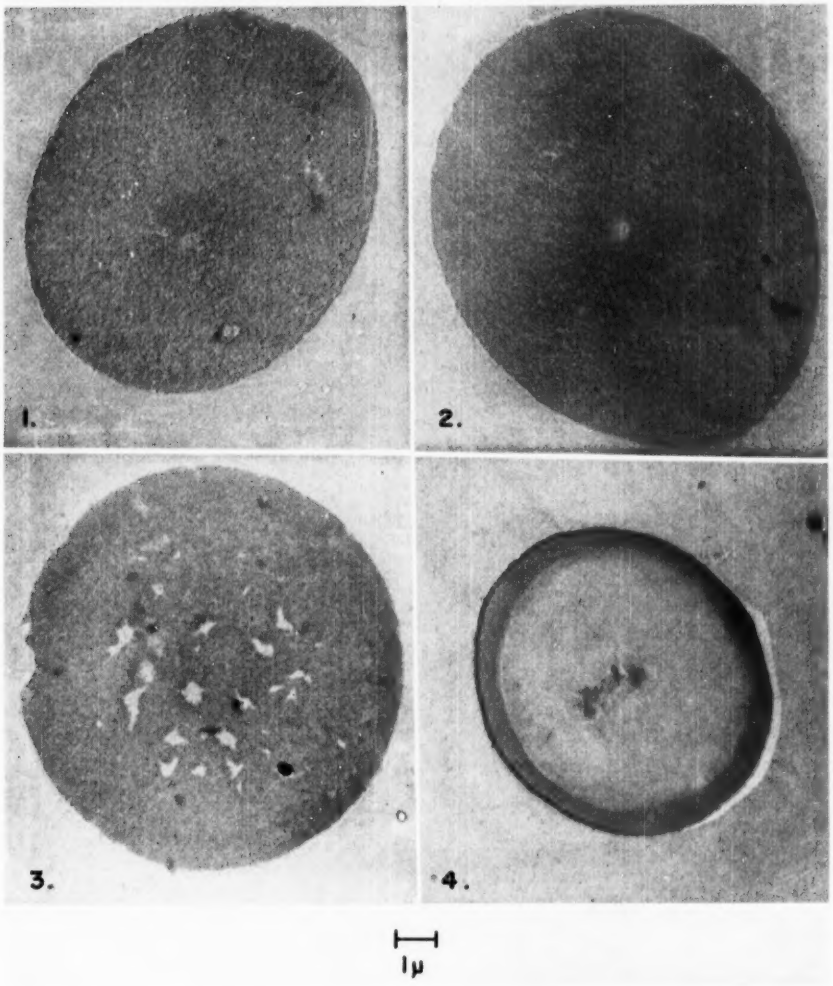
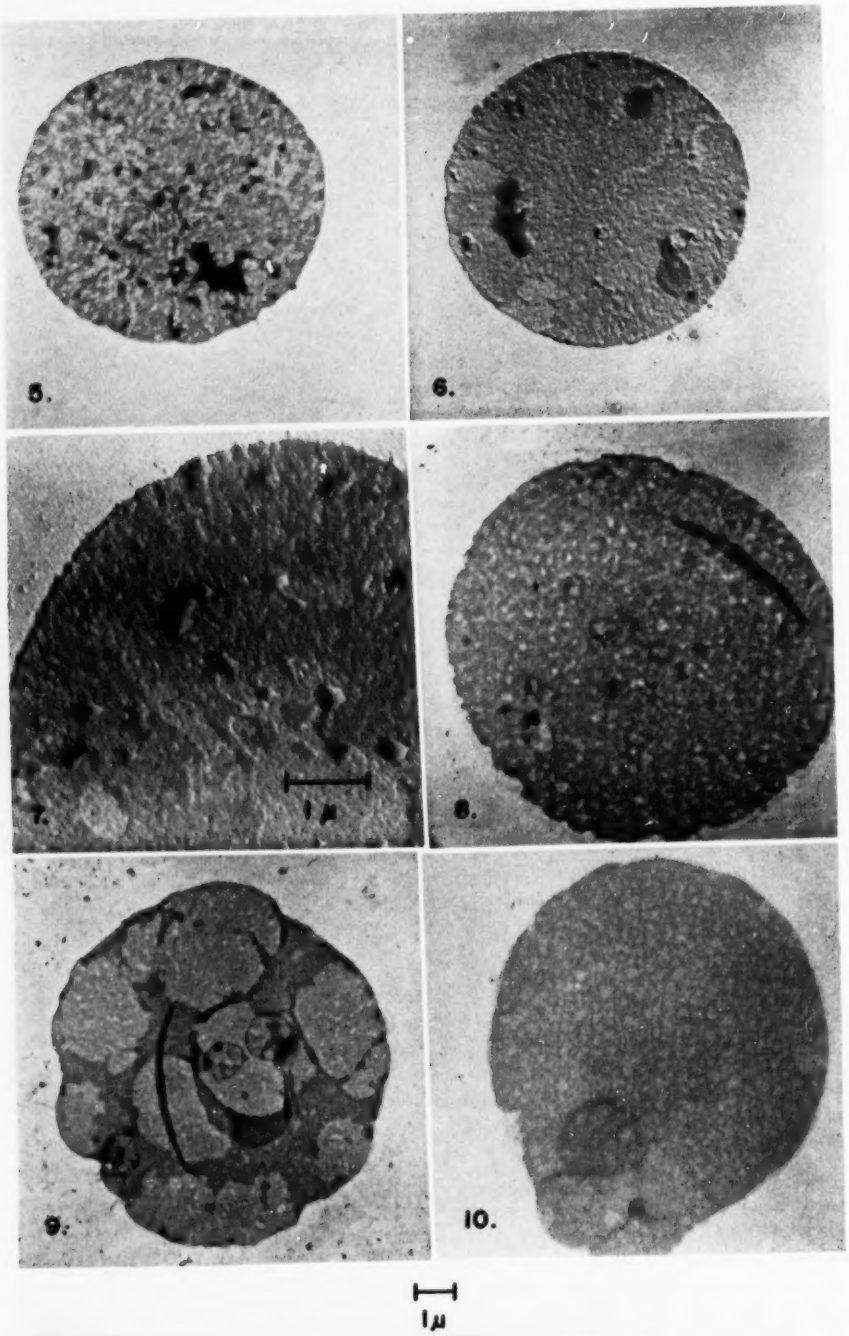


PLATE I

FIG. 1. NORMAL ERYTHROCYTE MEMBRANE (GOLD SHADOWING, 10 MG. 12 INCHES, ANGLE 11°)
FIG. 2. NORMAL ERYTHROCYTE MEMBRANE (GOLD SHADOWING, 10 MG. 12 INCHES, ANGLE 11°)
FIG. 3. NORMAL ERYTHROCYTE MEMBRANE (WITHOUT SHADOWING)
FIG. 4. PARTIAL HEMOLYZED ERYTHROCYTE WITH RESIDUAL HEMOGLOBIN IN THE PERIPHERY (GOLD SHADOWING, 10 MG. 12 INCHES, ANGLE 11°)

PLATE II



II. *Erythrocyte membranes from cases of hemolytic syndromes, leukemia, polycythemia vera, malignant diseases, tuberculosis, and iron deficiency anemia*

A. *Hemolytic anemia*

1. *Acquired hemolytic anemia.* Twenty-three specimens from 11 cases of acquired hemolytic anemia were studied. In every case, incomplete antibodies were demonstrated by the anti-human globulin (Coombs) test and/or by the trypsinized red cell test. Changes were observed in 10 to 50 per cent of the erythrocyte population in 9 of the 23 specimens. These changes consisted of varying degrees of thickening of the membrane accompanied by irregular hole formation. Hemoglobin fragments were frequently attached to the surface (Figures 5, 6 and 7). When multiple examinations of membranes from a single case of acquired hemolytic anemia were repeated over a period of months, the same changes were constantly observed.

2. All of the five cases of spherocytosis had undergone splenectomy several years previously. Separation and recognition of spherocytic membranes were less effective than in the cases of acquired hemolytic anemia.

3. The two specimens from the single case of thalassemia major showed the changes which were qualitatively and quantitatively similar to those in acquired hemolytic anemias (Figure 8).

4. Four specimens from two cases of mild sickle cell anemia showed no abnormalities.

B. *Leukemia*

In only 9 of 55 cases of chronic leukemia were modifications in the erythrocyte membranes evident. In eight of these cases, 10 to 30 per cent of the membranes showed changes, while in a case associated with frank intermittent hemolytic episodes, 90 per cent of the membranes appeared abnormal. Large holes with a diameter equal to one-third of that of the membrane were frequently noted (Figure 9). The impression gained was that many of these ultrastructures had marked weakness of the wall and a high susceptibility to even slight trauma. In 11 of the 55 cases, fold formations were noted in 5 to 15 per cent of the total membranes.

C. *Polycythemia vera*

Five specimens from four cases were examined. In two of these the membranes showed numerous small, irregular holes in less granular than normal stroma (Figure 10). This particular pattern was not seen in any of the other clinical or experimental material.

D. *Malignant diseases and tuberculosis*

No abnormal changes were noted in the membranes from 24 cases of these diseases.

E. *Iron deficiency anemia*

A thin fold formation was evident in 10 per cent, 50 per cent, and 90 per cent of the erythrocyte membranes in three cases of iron deficiency anemia (Figure 11). In the case with 90 per cent fold formation, five weeks of iron therapy effected an elevation in both the erythrocyte and hemoglobin levels with a diminution in the incidence of fold formation to 70 per cent. Unfortunately, further follow-up on this patient was not possible.

III. *Modified erythrocytes*

A. *The fold formation of the membrane* as first observed in the iron deficiency anemias, stimulated our interest. Previously, several investigators

PLATE II

FIG. 5. ERYTHROCYTE MEMBRANE OF THE SPHEROCYTE FROM ACQUIRED HEMOLYTIC ANEMIA (WITHOUT SHADOWING)

FIG. 6. MEMBRANE OF THE SPHEROCYTE FROM THE SAME SAMPLE OF FIG. 5 (CHROMIUM SHADOWING, 31 MG. 12 INCHES, ANGLE 18°)

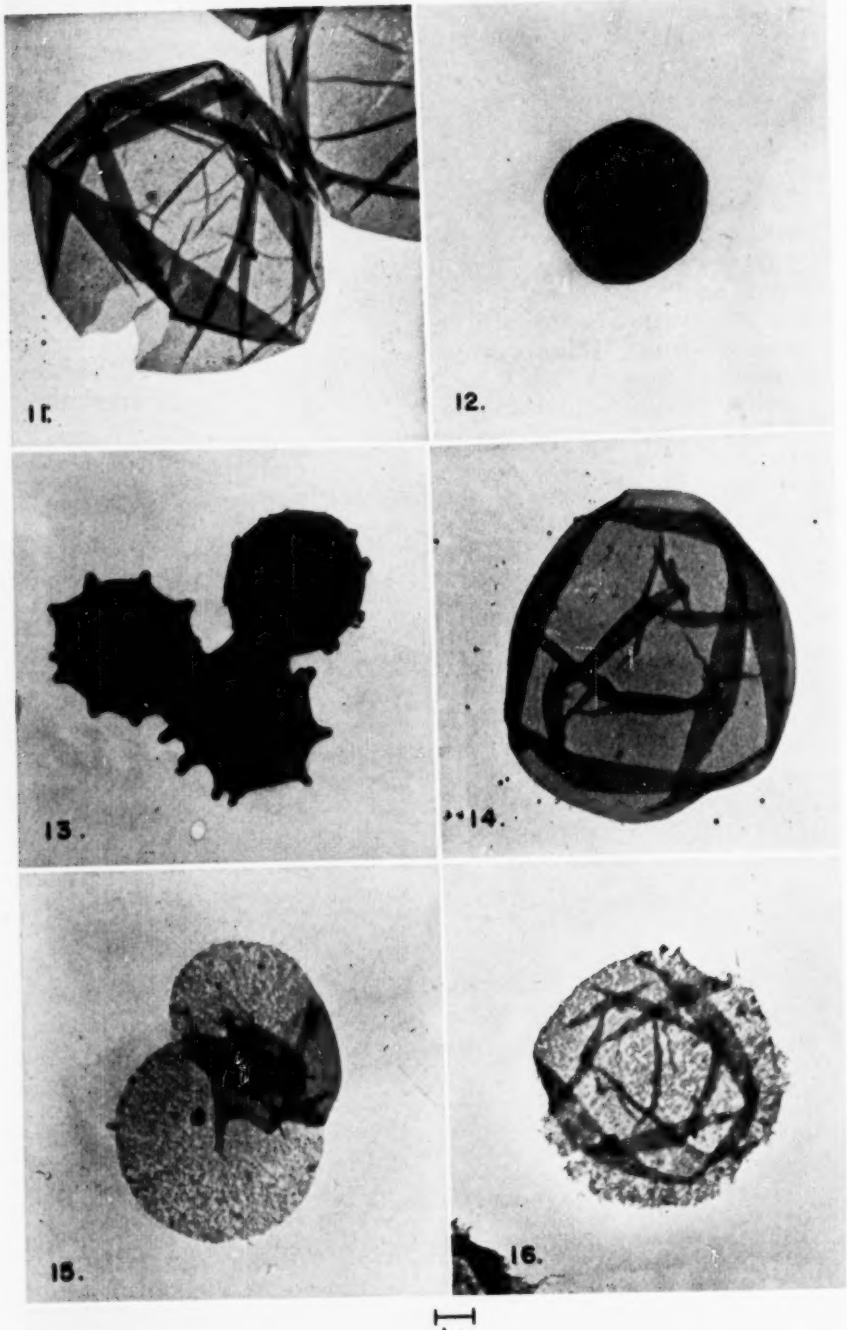
FIG. 7. MEMBRANE OF THE SPHEROCYTE (HIGHER MAGNIFICATION, CHROMIUM SHADOWING, 31 MG. 12 INCHES, ANGLE 18°)

FIG. 8. MEMBRANE FROM A CASE OF THALASSEMIA MAJOR (WITHOUT SHADOWING)

FIG. 9. MEMBRANE FROM A CASE OF CHRONIC MYELOID LEUKEMIA (WITHOUT SHADOWING)

FIG. 10. MEMBRANE FROM A CASE OF POLYCYTHEMIA VERA (WITHOUT SHADOWING)

PLATE III



1 μ

noted fold formation in all of the specimens they examined; however, they reported using osmic acid or formalin as fixatives (12–14). Since fixatives denature the surface protein, the elasticity of the membrane may be destroyed. It seemed pertinent to the problem to study the effects of these agents as well as those of marked lowering of the pH and other fixative agents which produce similar denaturing effects.

Osmic acid 1 per cent—All the membranes treated showed fold formation. When normal intact erythrocytes were treated, there was no hemolysis; however, there was marked contraction of the entire cell to approximately half the normal diameter (Figure 12). Some cells similarly treated showed crenation and agglutination (Figure 13). Folds were not present in the wet preparations of erythrocyte membranes when observed under the light microscope, but were evident after drying (Figure 14). In another experiment, membranes on a wet screen were exposed to osmic acid vapor and showed fold formation after drying. Membranes dried previous to treatment with the vapor showed no folding. These observations suggest that the folds developed during the drying process.

Formalin 1 per cent and 10 per cent—Membranes treated with formalin showed folding and coarse granulation suggestive of a coagulation effect of the surface structure (Figure 15).

Salts of heavy metals—All membranes treated with copper sulfate, lead nitrate, ferric sulfate, mercuric chloride and silver nitrate showed varying degrees of folding (Figure 16). Intact erythrocytes treated with mercuric chloride and silver nitrate showed no hemolysis; there was contraction with some shifting of the internal hemoglobin and "cracking" of the intact cells (Figure 17). Ferric chloride treatment produced a swirling type of fold (Figure 18).

Hemolysis of normal erythrocytes in distilled water of various pH—Marked fold formation in the membranes resulted from treatment with distilled water at pH 3.0 (Figure 19). At pH 1.5, folds were delicate and numerous (Figure 20). At pH 10.4, there was marked stromatolysis with stringlike formation (Figure 21). Hemolysis of a sample at pH 11.0, and subsequent lowering of the pH to 7.0, produced fragmentation of the membrane, with interspersed masses of hemoglobin (Figure 22). Normal membranes were subjected to distilled water with a pH of 1.5 for 10 minutes. The membranes showed disintegration similar to, but not as severe as, that obtained at pH 11.0 and a subsequent correction to a pH of 7.0.

IV. *Trypsinized erythrocytes*

Trypsinized erythrocytes (*1*) have proved to be a useful tool in demonstration of incomplete antibodies (15); (*2*) are more easily phagocytized than normal erythrocytes (16); and (*3*) have a shortened life span in the experimental laboratory animal (17). These characteristics led to their inclusion in this study.

Twenty-eight specimens of trypsinized red cells were examined. Two methods of trypsinization were employed: 1) treatment of the cells in suspension and 2) treatment after the membranes had been "filmed" on the Formvar screen. Various concentrations from 0.1 per cent to 4 per cent trypsin were used.

Membranes prepared from red blood cells treated by the first method showed no change from normal when the lower concentrations of trypsin were used. Only when cells were tryp-

PLATE III

FIG. 11. ERYTHROCYTE MEMBRANE FROM A CASE OF IRON DEFICIENCY ANEMIA (WITHOUT SHADOWING)

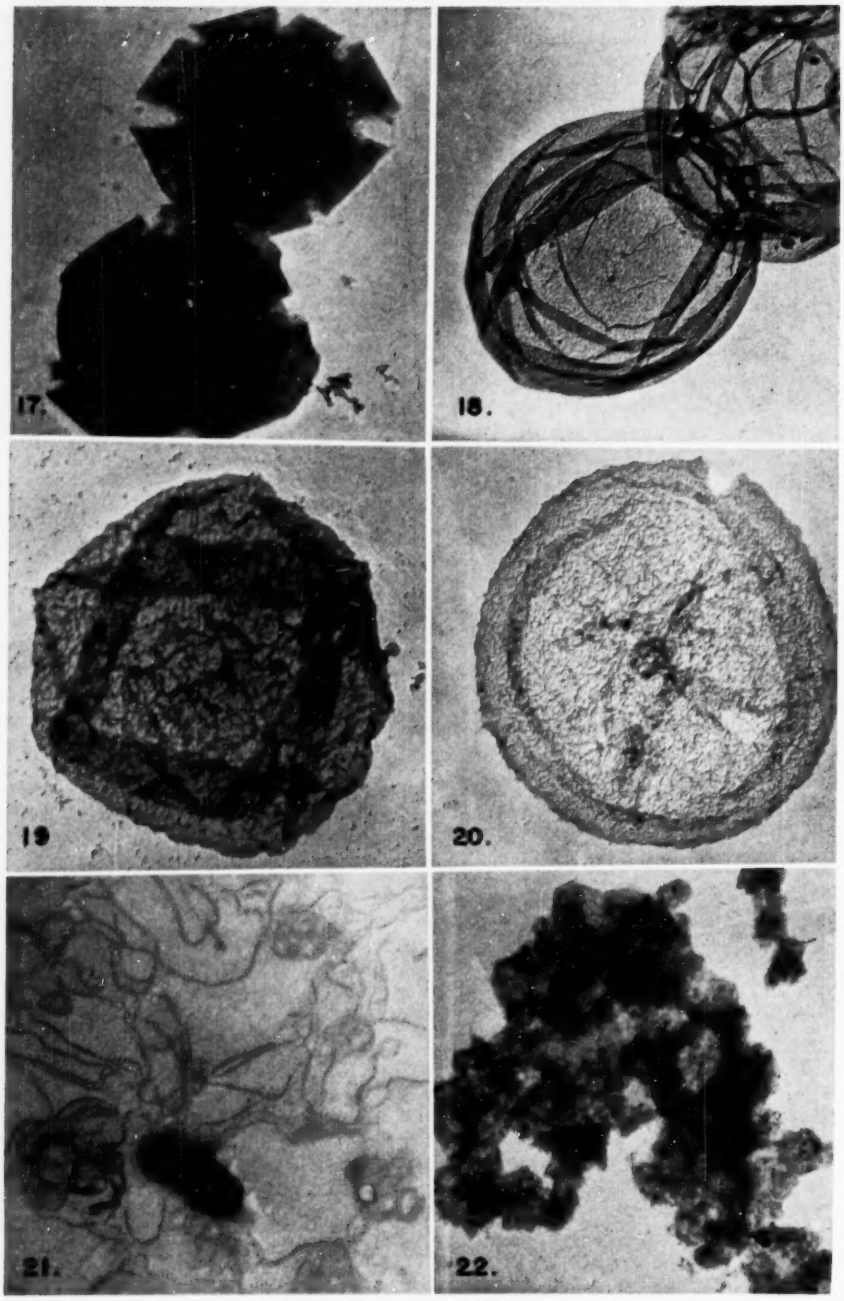
FIGS. 12–13. NORMAL ERYTHROCYTES TREATED WITH 1.0 PER CENT OSMIC ACID, 10 MIN. (WITHOUT SHADOWING)

FIG. 14. NORMAL ERYTHROCYTE MEMBRANE TREATED WITH 1.0 PER CENT OSMIC ACID, 10 MIN. (GOLD SHADOWING, 10 MG. 12 INCHES, ANGLE 11°)

FIG. 15. NORMAL ERYTHROCYTE MEMBRANE TREATED WITH 10.0 PER CENT FORMALIN 10 MIN. (GOLD SHADOWING, 10 MG. 12 INCHES, ANGLE 11°)

FIG. 16. NORMAL ERYTHROCYTE MEMBRANE TREATED WITH 1.0 PER CENT COPPER SULPHATE SOLUTION, 10 MIN. (GOLD SHADOWING, 10 MG. 12 INCHES, ANGLE 11°)

PLATE IV



sinized with 4 per cent trypsin solution did the membranes show modifications. Erythrocyte membranes from several cases of chronic leukemia which showed no evidence of clinical hemolysis were much more susceptible to trypsin treatment than were normal cells (Figure 23). Two per cent trypsin solution was used in these comparative studies.

Trypsin produced marked changes in the membranes even in a low concentration (0.1 per cent) if the membranes were dried before treatment. The changes were in proportion to the concentrations of trypsin (Figure 24).

V. *Sensitized erythrocytes*

Sensitized erythrocytes showed crenation and agglutination under the light microscope. In the 35 specimens studied, various abnormalities were manifested (Figure 25). Following weak sensitization the membranes appeared thinner than normal and showed some folding. Strong sensitization increased coarseness and granular changes.

VI. *Irradiated erythrocytes*

A. Thirty specimens from rabbits which had received 850 to 900r total body irradiation showed no change from the normal.

B. Thirty-two specimens of *in vitro* irradiated erythrocytes were studied. In only one, irradiated with 48,000 r, were variations consisting of coarse granulation and cracks noted (Figure 26). These findings are at variance with those of Zacek and Rosenberg (18) who noted marked ultrastructure modifications. They hypothesized that the irradiation caused changes in the lipoprotein. Halberstaedter and Goldhaber (19) demonstrated changes with irradiation as noted with chemical studies; they also claimed that changes were dependent on the concentration of erythrocytes and the character of the suspension medium. Our technic was identical with that of Zacek and Rosenberg with the exception that physiologic saline was substituted for Ringer's solution. The Halberstaedter and Goldhaber concentrations were less than the 1 : 1 suspensions used here.

VII. *Stored erythrocytes*

Stored erythrocytes were studied in 43 specimens. No correlation between the changes and the duration of storage was demonstrated. Cells stored for 120 days did not show significant modifications. The few membranes that did show changes demonstrated a thinner periphery. This observation is similar to previous leptoscopic study of normal cells which showed that the ultrastructure in the region of the biconcavities was thicker than that of the periphery (1) (Figure 27).

VIII. *Influence of temperature on normal erythrocyte membranes*

A. Red cells incubated under sterile conditions in both plasma and defibrinated blood for varying periods from ½ to 24 hours at 37° C. showed no changes in their membranes. It was thought that minimal differences might be detected inasmuch as 24 hours incubation did produce differences in the osmotic fragilities of erythrocytes from acquired hemolytic anemia and hereditary spherocytosis.

B. Erythrocytes in saline heated for 30 minutes at 56° C. showed "coagulation" and disintegration of their membranes with hemoglobin attached (Figure 28).

PLATE IV

FIG. 17. NORMAL ERYTHROCYTE TREATED WITH 1.0 PER CENT MERCURIC CHLORIDE SOLUTION, 10 MIN. (GOLD SHADOWING, 10 MG. 12 INCHES, ANGLE 11°)

FIG. 18. NORMAL ERYTHROCYTE MEMBRANE TREATED WITH FERRIC CHLORIDE 0.01 M SOLUTION FOR 10 MIN. (GOLD SHADOWING, 10 MG. 12 INCHES, ANGLE 11°)

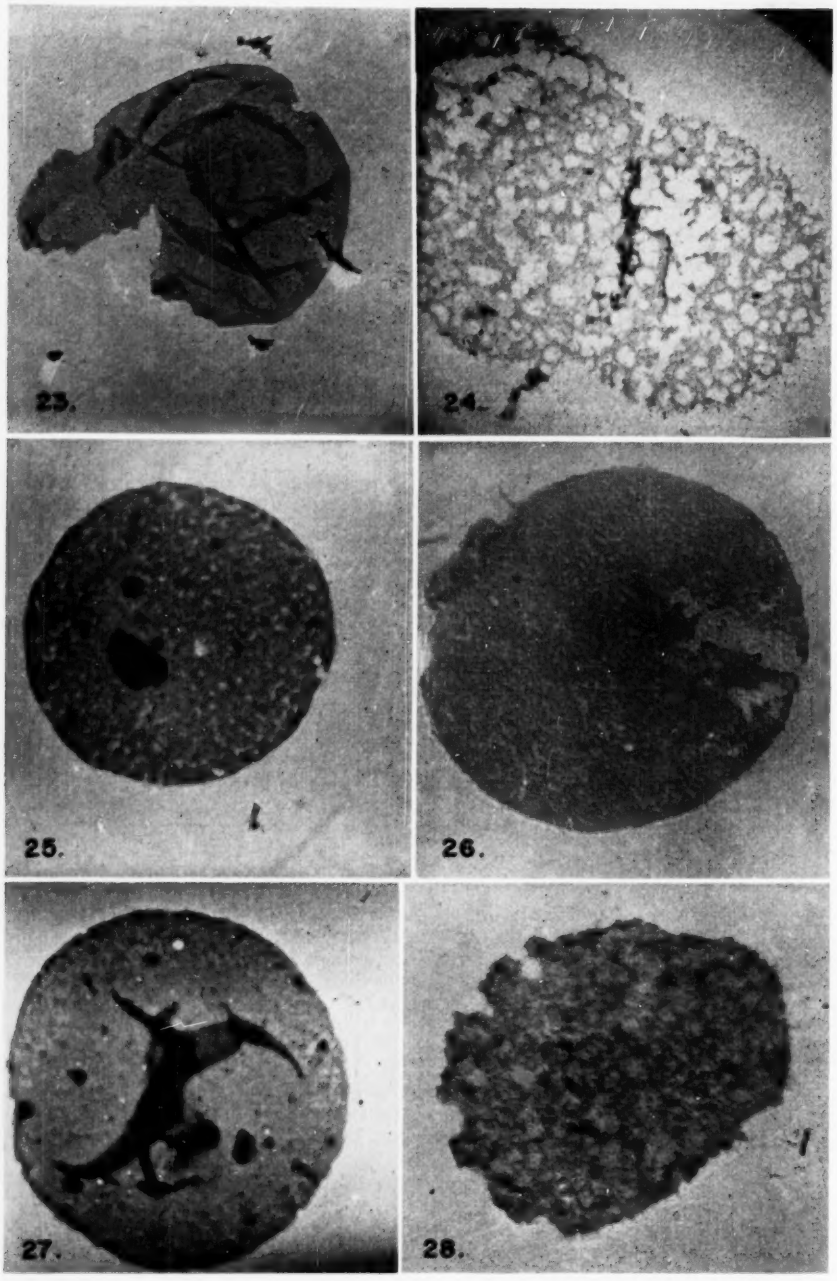
FIG. 19. MEMBRANE FROM NORMAL ERYTHROCYTE HEMOLYZED IN DISTILLED WATER pH 3.0 FOR 10 MIN. (GOLD SHADOWING, 10 MG. 12 INCHES, ANGLE 11°)

FIG. 20. MEMBRANE FROM NORMAL ERYTHROCYTE HEMOLYZED IN DISTILLED WATER pH 1.5 FOR 10 MIN. (GOLD SHADOWING, 10 MG. 12 INCHES, ANGLE 11°)

FIG. 21. MEMBRANE FROM NORMAL ERYTHROCYTE HEMOLYZED IN DISTILLED WATER pH 10.4 FOR 10 MIN. (GOLD SHADOWING, 10 MG. 12 INCHES, ANGLE 11°)

FIG. 22. MEMBRANE FROM NORMAL ERYTHROCYTE HEMOLYZED IN DISTILLED WATER pH 11 FOR 10 MIN., THEN THE pH WAS CORRECTED TO 7 (GOLD SHADOWING, 10 MG. 12 INCHES, ANGLE 11°)

PLATE V



C. Erythrocytes hemolyzed in distilled water at 56° C. for 10 minutes also demonstrated the "coagulation" phenomena and disintegration.

D. Membranes suspended in distilled water and heated at 56° C. for 30 minutes showed changes similar to those observed in the two above conditions.

E. Storage of normal membranes in distilled water (pH 6.5) at 4° C. for 21 days occasioned no change from the normal.

COMMENTS

In assessing the importance of the observations reported here, care must be taken to avoid absolute correlations and conclusions based on phenomena which may be essentially of an artifactual nature. Even if they are artifactual, it is quite possible that they may reflect some other acquired or inherited phenomena. The quantitative differences that were seen in much of the material from pathologic sources when compared with the control material from normal healthy sources do allow for a certain degree of hypothesizing. The following hypothesis as to the folding mechanism is offered: During the process of hemolysis, osmosis causes some distention of the red cell. Normally, the ultrastructure is resilient, and there is contraction before drying is complete. If the elasticity is destroyed, (as by fixatives, sensitization, trypsinization or treatment with salts of the heavy metals) or is inherently diminished (as may be in iron deficiency anemia) there is failure of contraction and the membranes fold together on the surface with the folds becoming visible. The high percentage of folding in some of the clinical material (which received identical preparation as the normal controls) could influence one to project the possibility that fold formation could be due to a defective ultrastructure architecture.

Similarly, the significance of holes in the membranes is uncertain. Although they, as in the case of folds, are present in a small percentage of normal erythrocytes, their marked occurrence in several cases of acquired hemolytic anemia, thalassemia major, and chronic leukemia suggests a possible role in the hemolytic process. The question arises: Is hemoglobin expelled through holes? Sasagawa, Hosomi, and Mani (10), after observing the process of hemolysis with the phase contrast microscope, concluded that hemoglobin may be liberated by several means: (1) a large crack in the membrane (as seen in Figure 11); or (2) by bursting out of one or a few "spots" on the membrane (as in Figures 5, 6 and 7); or (3) by transudation out of the whole surface of the cell through numerous "pores" (as in Figure 10). It is evident, however, that further studies are necessary before such correlations can be soundly made.

SUMMARY

The erythrocyte ultrastructure membranes from normal individuals, individuals suffering from various hematologic diseases and from modified erythrocytes were studied with the electron microscope. The following observations were made:

1. Normal erythrocyte membranes showed a finely granular surface. Only rarely were holes or fold formations (1 to 3 per cent) evident.

2. Many of the red cell ultrastructures from cases of acquired hemolytic anemia, hereditary spherocytosis, thalassemia major, chronic leukemia, and polycythemia vera showed varying modifications such as increased coarseness and hole and fold formation.

PLATE V

FIG. 23. MEMBRANE OF NORMAL ERYTHROCYTE MODIFIED BY 4.0 PER CENT TRYPSIN (CHROMIUM SHADOWING, 31 MG. 12 INCHES, ANGLE 18°)

FIG. 24. DRY NORMAL ERYTHROCYTE MEMBRANE DIGESTED BY 0.5 PER CENT TRYPSIN (WITHOUT SHADOWING)

FIG. 25. MEMBRANE OF NORMAL ERYTHROCYTE MODIFIED BY SENSITIZATION (WITHOUT SHADOWING)

FIG. 26. ERYTHROCYTE MEMBRANE AFTER IRRADIATION 48,000 r (GOLD SHADOWING, 10 MG. 12 INCHES, ANGLE 11°)

FIG. 27. MEMBRANE FROM STORED ERYTHROCYTE (SEVEN DAYS) (WITHOUT SHADOWING)

FIG. 28. MEMBRANE FROM HEATED ERYTHROCYTE IN NORMAL SALINE 56° C. FOR 30 MIN. BEFORE HEMOLYSIS (GOLD SHADOWING, 10 MG. 12 INCHES, ANGLE 11°)

3. The fold formations that were seen in many membranes from patients with iron deficiency anemia, and chronic leukemia led to an experimental study of the production of fold formation. Experimentally, fixatives such as formalin and osmic acid, salts of heavy metals, lowering of the pH below 3.0, intense sensitization and trypsinization also produced folding of the ultrastructure. Similar treatment also produced varying degrees of coarseness, cracking, holes, and disintegration.

4. Irradiated erythrocytes *in vivo* showed no change. In only one of thirty-two specimens irradiated *in vitro* did the membranes show modifications. Stored erythrocytes and erythrocytes incubated at 37° C. for 24 hours also showed no change.

5. The significance and interpretation of the modifications observed in both the clinical and experimental material must be guarded pending further study.

ACKNOWLEDGMENTS

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THE DETERMINATION OF PLASMA VOLUME IN MAN WITH RADIOACTIVE CHROMIC CHLORIDE [1]

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Radioactive chromium has been found to tag red blood cells and plasma proteins (1). The anionic form, $Na_2Cr^{51}O_4$, labels the red blood cells, while the cationic hexavalent chromic chloride is firmly bound by the plasma proteins and is not taken up by the red cells. The affinity of the erythrocytes for sodium chromate has been used to measure the circulating red cell mass in man (2, 3).

Radioactive chromic chloride is rapidly bound by the plasma proteins both *in vivo* and *in vitro*, and the binding appears to be relatively stable (1). The utilization of radioactive chromic chloride in the determination of plasma volume was suggested by the observation that 98 per cent or more of the chromic chloride is bound immediately by the plasma proteins when injected intravenously into humans. Once the chromic chloride is bound to the plasma proteins, it can leave the circulation only at the slow rate at which the proteins leave it. The dilution of this tracer substance in the circulation after intravenous administration was, therefore, used to measure the plasma volume in man.

The plasma volume determination using radioactive chromic chloride presents a simple, accurate, and rapid technique, by which a stable, non-toxic chemical solution can be injected directly, obviating the necessity of tagging albumin or other proteins *in vitro*. Determinations can safely be performed repeatedly and in rapid succession. Self-absorption corrections are not necessary. Liquid phase gamma ray counting permits the measurement of the plasma volume within a few hours.

The plasma volumes of twenty-six normal adults have been determined by this method. In some of the subjects, a second determination has been done days to weeks later. The method was further tested by measuring the plasma volume before and after transfusion or hemorrhage of a known volume of plasma in hospital patients and volunteer subjects.

[1] This work was supported in part by the United States Atomic Energy Commission and the United States Public Health Service.

METHODS

I. Preparation of chromic chloride

Radioactive chromic chloride is prepared from $Na_2Cr^{51}O_4$. The preparation of $Na_2Cr^{51}O_4$ from $Cr^{51}O_3$ has been described previously (1).[2]

$Na_2Cr^{51}O_4$, containing 25 to 30 mg. of chromium, is dissolved in 50 ml. of water in a 200 ml. beaker. Glass beads are added. Six ml. of concentrated HCl are added, turning the solution orange. When 6 ml. of 37 per cent formaldehyde are added the solution becomes green. This green solution is evaporated to dryness on a hot plate at 700° C. The solution must be shaken constantly while on the hot plate to prevent bumping. When evaporation is near completion, green crystals [3] form, which become light violet when complete dryness is reached. The beaker is removed from the hot plate as soon as the green crystals begin to turn violet and the crystals then resume their green color. Thirty-five to 40 ml. of water are added to the beaker to dissolve the crystals. The resulting green solution is filtered through No. 3 Whatman filter paper into a 50 ml. volumetric flask. This filtration should be done even though the solution looks clear. The pH of the solution is adjusted to 4.1 by the addition of 0.5 normal NaOH. If the pH is too high, $Cr(OH)_3$ will precipitate out. This can be corrected by the addition of a very small amount of HCl. The green solution in the volumetric flask is brought up to volume with water. It is stable at room temperature for several months.

[2] $Na_2Cr^{51}O_4$ is now obtainable from Abbott Laboratories.

[3] The crystals so obtained are probably a mixture of the hexahydrated forms of chromic chloride $(CrCl_2(H_2O)_4)Cl \cdot 2H_2O$, $(CrCl(H_2O)_5)Cl_2 \cdot H_2O$, and $(Cr(H_2O)_6)Cl_3$. Anhydrous chromic chloride is purple and quite insoluble. From pilot experiments it seems that not all the hexahydrates are equally efficient in tagging plasma proteins. A blue solution containing a preponderance of $(Cr(H_2O)_6)Cl_3$ does not tag as well as a green solution. Work is in progress to determine quantitatively the protein binding capacity of the different forms. For practical purposes, the crude color index seems adequate and the green solutions prepared by the method described have invariably tagged the proteins well.

II. Dosage

Electromagnetically enriched Cr^{50}, irradiated in the Oak Ridge pile for two months, yields Cr^{51} with a specific activity of about 0.6 microcurie per microgram chromium. The chromium obtainable through Abbott Laboratories also has a specific activity of 0.5 to 0.7 microcurie per microgram chromium.

The radiation dosage is calculated by the formula of Marinelli, Quimby, and Hine (4), assuming that 10 per cent of the activity of Cr^{51} is due to gamma rays and that the half-life is 26.5 days (see Appendix A). The injection of the usual dose of 100 microcuries Cr^{51} into an individual weighing 70 Kg. represents a total radiation dose of 0.1 rep. Since the safety limits for human tracer studies ar 0.3 rep. per week, repeated administration is permissible.

The amount of chromium in the usual dose of 100 microcuries varies from 0.1 to 1.0 mg. or 1 to 14 gamma per kilogram of body weight, depending on the specific activity of the material. This is well below the toxic level.

III. Experimental procedure

A. Injecting and sampling procedure.

Approximately 100 microcuries of chromic chloride (0.1 to 1.0 mg. chromium) are suspended in 30 ml. of normal saline. This solution is autoclaved at 250° C. and 18 pounds pressure for ten minutes and then allowed to stand in the autoclave for another ten minutes. More intense heat and more prolonged heating seem to decrease the protein binding ability of the chromic chloride. Twenty-five ml. of the solution are drawn into a calibrated syringe, and the remainder is saved for counting.

A 10 ml. sample of blood is withdrawn from the subject into a heparinized syringe as the control sample. Syringes are changed and the chromic chloride solution is injected through the same needle. After allowing five minutes for mixing within the circulation, four samples of venous blood are drawn from the opposite arm at five minute intervals for twenty minutes. The time intervals are accurately established with a stop watch.

All samples are collected in heparinized syringes. The withdrawals are made from the opposite arm to that used for injection. Hemostasis is avoided as much as possible by releasing the tourniquet as soon as the needle is in the vein. Subjects need not be in the fasting state.

B. Counting procedure.

Radioactive chromium can be counted in the liquid or the dried state. For liquid sample counting two types of counters have been used in this laboratory: a well-type gamma ray counter (Texas Co., type H18-20 TR)[4]; and a liquid sample scintillation counter (W. S. McDonald Co., Inc., counter type 155, detector head type 152-H5). One microcurie of Cr^{51} records approximately 5900 counts per minute on the well-type counter and 65,170 counts per minute on the scintillation counter (Table I).

[4] We should like to acknowledge our indebtedness to the Texas Company for supplying this counter.

TABLE I

The counting efficiencies for Cr^{51} of several counters

Counter	Mean efficiency	Mean corrected counts *per minute μc.*	Background *counts per minute*
McDonald scintillation counter	2.96	65,170	450
Texas Company well counter	.3	5,900	390
Robinson flow counter	23.6	519,450	20
End window tracerlab tube	.8	17,120	18
End window amperex tube	.5	11,770	10

When radioactive chromium is to be counted in the dried state, an x-ray end window Geiger-Muller counter or a windowless flow type proportional counter can be used. The two end window tubes employed in this study were the North American Phillips, tube type 62017-Amperex, giving 11,770 counts per microcurie Cr^{51} per minute, and the Tracerlab. Inc., tube TCG 3, giving 17,-120 counts per microcurie Cr^{51} per minute. The windowless flow type proportional counter (modification of the counter described by C. V. Robinson [5]) records approximately 519,450 counts per microcurie Cr^{51} per minute.

The plasma samples obtained after the injection of the usual dose of 100 microcuries of Cr^{51} will register approximately 150 to 200 counts per ml. in one minute on the end window Amperex tube. Our routine procedure is to count at least a total of 1300 counts, requiring in this case a counting time of 7 to 9 minutes per sample to keep the probable counting error within 2 per cent. Using the Tracerlab end window counter or the flow counter, the counting time is even shorter because of their higher efficiencies (see Appendix A). On both liquid sample counters, because of their higher backgrounds, a total of 10,000 counts is usually counted, requiring approximately five minutes counting time on the scintillation counter and fifteen minutes on the well counter.

C. Preparation of samples.

All blood samples are centrifuged in regular 15 ml. conical centrifuge tubes at 2700 rpm. at a radius of 14 cm. for one hour, and the plasma is separated. If dried samples are to be counted, one ml. portions of each plasma sample are pipetted into aluminum planchets. The planchets are prepared in duplicate.

The counts contained in the chromic chloride solution, which is injected into the subject, are determined by diluting an aliquot of the saline solution 1 : 50 with distilled water. Five-tenths ml. aliquots of this diluted solution are pipetted into duplicate planchets. Self-absorption corrections can be eliminated by adding one ml. of the subject's control plasma to the 0.5 ml. aliquot. The plasma and the chromic chloride solution are mixed with a clean wire in the planchets.

All samples are dried over-night in air, or for about four hours in an oven at 60° C. At higher temperatures, chromium may be lost by sublimation. The samples are then stored in a desiccator until they are counted.

For liquid sample counting in the well type gamma ray counter, volumes of 6 to 7 ml. can be counted in ordinary thin-walled 15 ml. conical centrifuge tubes. In the scintillation counter 2 ml. samples are counted in small rimless culture tubes. No absorption corrections are necessary in these counters.

IV. Calculations

In the plasma volume determination with radioactive chromium, as with all other methods for plasma volume now available, the dilution curve is not "flat," and extrapolation to zero time is necessary (6). Mixing of the injected material with the blood is complete within five to ten minutes after injection (7). By obtaining four plasma samples at five minute intervals after the injection of chromic chloride and plotting the counts per ml. of plasma against time on semi-logarithmic paper, a straight line is obtained and the theoretical counts per ml. of plasma at zero time can be extrapolated. With this value, the plasma volume is calculated by the formula:

$$\text{Plasma Volume} = \frac{\text{Total counts injected} - 2 \text{ per cent}}{\text{Counts/ml. plasma at zero time}}$$

The total counts injected are calculated as the product of the number of counts per ml. of the diluted chromic chloride solution, the dilution factor, and the volume of chromic chloride injected (Appendix B). The 2 per cent subtracted from the total counts injected represent the average maximum counts lost to the erythrocytes (Table II).

Whenever the plasma volume determination is repeated, such as following hemorrhage or transfusion, a second dose of chromic chloride is administered. A significant number of counts may still be present in the plasma at the time of the second injection of chromic chloride.

TABLE II

Loss of radioactive chromic chloride to the red cells

Subject	Counts injected $\times 10^6$	Counts per ml. RBC's	Counts on RBC's $\times 10^4$	Per cent counts on RBC's
P. D.	24.8	193	42.4	1.7
J. McV.	22.2	365	80.5	3.6
W. T.	20.6	159	35.0	1.7
R. R.	24.3	379	83.4	3.4
L. G.	25.4	287	63.1	2.5
J. P.	25.8	172	37.8	1.5
J. G.	26.2	177	38.9	1.5
D. G.	21.6	291	64.0	2.9
C. H.	21.8	160	35.2	1.6
R. P.	105.1	449	98.8	0.9
B. B.	91.7	326	71.7	0.8
M. R.	100.1	839	184.6	1.8
P. C.	95.3	342	75.2	0.8
M. P.	38.0	562	123.6	3.2
L. S.	18.7	57	12.5	0.7
			Average	1.9
			Standard Deviation	±0.96
			Standard Error of Mean	0.25

In the calculation of the final plasma volume, these retained counts are subtracted from the counts in the plasma after extrapolation to zero time to obtain the corrected counts (Appendix C).

$$\text{Second Plasma Volume} = \frac{\text{Total counts in 2nd injection} - 2 \text{ per cent}}{\text{Corrected counts/ml. plasma at zero time}}$$

RESULTS

Plasma volume determinations with radioactive chromic chloride were performed on 26 normal adults (Table III). The mean plasma volume for the 21 males was 2894 ± 366 ml. by this method. When calculated on the basis of body weight, the mean plasma volume was 39.3 ± 4.9 ml./Kg. by the radioactive chromium technique, as compared to the value of 43.08 ± 5.9 ml./Kg. with Evans blue dye obtained by Gibson and Evans (8), and the Holden value of 40.6 ± 4.4 ml./Kg. with radioactive iodinated plasma obtained by Storaasli,

TABLE III

Circulating plasma volumes in twenty-one adult males and five females

Subject: Male	Wt. *Kg.*	Ht. *cm.*	Surface area *sq. m.*	Total P.V. *ml.*	Plasma volume *ml./Kg.* of body weight	Plasma volume *ml./Sq. M.* Surface area
P. D.	70	166	1.76	2830	40.4	1610
J. McV.	64	190	1.88	2930	45.8	1560
W. T.	61	170	1.70	2960	48.5	1740
R. M.	80	189	2.05	3180	39.7	1550
D. P.	68	178	1.83	3010	44.3	1640
R. P.	71	174	1.84	2530	35.6	1375
B. B.	88	180	2.07	3030	34.4	1460
M. R.	77	174	1.90	2210	28.7	1165
P. C.	62	178	1.76	2740	44.2	1555
J. McK.	82	186	2.04	3330	40.6	1630
S. P.	66	186	1.86	2750	41.3	1480
J. T.	85	186	2.09	3000	35.3	1425
L. F.	80	186	2.03	3190	39.9	1570
L. G.	68	188	1.90	3010	44.3	1580
M. P.	87	179	2.04	3570	41.0	1750
R. C.	65	166	1.71	2140	32.9	1250
J. P.	77	187	2.00	2680	34.8	1340
F. H.	79	178	1.95	3400	43.0	1740
W. W.	77	180	1.90	2680	34.8	1410
T. D.	66	173	1.78	2470	37.4	1390
D. McD.	83	172	1.96	3130	37.7	1600
			Average	2894	39.3	1515
			Standard Deviation	±366	±4.9	±157
			Standard Error of Mean	80	1.1	34
Female						
E. P.	60	169	1.67	2380	39.6	1420
L. V.	57	168	1.65	1860	32.6	1130
E. C.	54	160	1.55	2190	40.5	1410
S. K.	55	158	1.55	1910	34.7	1230
M. C.	56	165	1.60	2120	37.8	1325
			Average	2092	37.0	1303
			Standard Deviation	±212	±3.3	±123
			Standard Error of Mean	95	1.4	55

TABLE IV

Plasma volume determination by radioactive chromic chloride before and after transfusion or hemorrhage

Patient	Sex	Weight Kg.	Initial P.V. ml.	Transfusion or hemorrhage ml.	Final P.V.			
					Calculated ml.	Measured ml.	Difference ml.	Difference (%)
R. C.	M	65	2144	−239	1905	1930	−25	−1.3
F. H.	M	79	3414	−240	3174	3260	−86	−2.7
M. C.	F	56	2116	+257	2373	2380	− 7	−0.3
W. J. W.	M	77	2676	+507	3183	3200	−17	−0.5
S. P.	F	43	2356	+550	2906	2833	+73	+2.5
H. D.	M	60	2159	+259	2418	2425	− 7	−0.3

Krieger, Friedell, and Holden (9). The values obtained by all three methods agree to better than one standard deviation. The correlation between the total plasma volume and the plasma volume related to body weight and surface area was calculated. The correlation coefficient between the total plasma volume and the plasma volume per square meter of surface area was 0.86, between the plasma volume per kilogram of body weight and that per square meter of surface area was 0.84. Both represent definitely significant correlations. The total plasma volume was somewhat less significantly related to the plasma volume per kilogram of body weight, with a correlation coefficient of 0.54.

In the five women studied, the plasma volume was lower than in the males, with a mean plasma volume of 2092 ± 212 ml. or 37.0 ± 3.3 ml./Kg. The figures of Gibson and Evans for women are 2284 ml. as the mean plasma volume or 41.5 ml./Kg. (8).

The accuracy of the method was verified by a second plasma volume determination immediately after the transfusion or hemorrhage of a known volume of plasma. The volume of plasma transfused or removed varied between 250 and 500 ml. The results of the second plasma volume determinations agreed with the expected values within 3 per cent (Table IV).

When the percentage error is calculated in terms of the actual volume of plasma lost or gained by the subject, the error is of necessity larger, averaging 11.3 per cent. This is inherent in the calculation and depends on the volume of plasma lost or gained and its proportion to the total plasma volume.

When 100 microcuries of radioactive chromic chloride are injected intravenously, only a very small fraction is lost to the erythrocytes. The radioactivity of the red cells was measured on all four blood samples drawn after the injection of chromic chloride in 15 subjects. The number of counts per ml. of washed red cells was measured and the counts in the total circulating red cell mass were calculated. The average loss of counts to the erythrocytes was 1.9 ± 0.9 per cent of the total counts injected (Table II). To correct for this small loss, 2 per cent of the counts were routinely subtracted from the total injected counts.

The amount of chromic chloride bound by the plasma proteins was determined by means of a sulfonated cationic exchange resin, Dowex No. 50,[5] in the sodium cycle, with a 20 to 40 mesh and 8 per cent cross linkage. The materials to be tested were run at a speed of 1 to 2 ml. per minute

[5] National Aluminate Corporation, Chicago 38, Illinois.

TABLE V

The binding of radioactive chromic chloride by plasma proteins

	Radioactive chromic chloride solution in saline	Plasma tagged *in vitro* with radioactive chromic chloride	Plasma tagged *in vivo* with radioactive chromic chloride
Corrected counts before passing through resin	9276	10276	635
Corrected counts after passing through resin	375	10150	633
Counts removed by resin	8901	126	2
Per cent counts removed by resin	95.6%	1.2%	.3%
Per cent of radioactive chromium bound to protein	—	98.8%	99.7%

through resin columns with a length of 10 cm. and a diameter of 1 cm. Under these conditions the resin removed 90 to 100 per cent of free chromic ion, within the range of concentration of chromic chloride used. When plasma, tagged with chromic chloride *in vivo* or *in vitro*, was passed through the resin, 98 to 100 per cent of the chromium was recovered, indicating that practically all of it was bound by the plasma (Table V).

The proteins of several plasma samples which had been tagged with radioactive chromic chloride *in vivo* were separated by fractional extraction (10). All the fractions showed radioactivity indicating that all fractions are tagged by radioactive chromic chloride.

In several normal subjects a second plasma volume determination, weeks to several months after the first one, agreed with the initial determination within 5 per cent.

DISCUSSION

The Cr^{51} method of determining the plasma volume in humans is based on the rapid *in vivo* binding of chromic chloride by the plasma proteins. All methods for the determination of plasma volume now available depend on the binding of the tracer substance to plasma proteins *in vivo* (8) or *in vitro* (9, 11–14), and are, therefore, open to the criticism that the plasma proteins do not constitute a well-defined compartment. However, although proteins do leave the vascular bed, the amount that escapes during the twenty-minute test period is probably too small to influence significantly the measurement of plasma volume (15).

The mean values for plasma volume obtained by the Cr^{51} method agree with those of other investigators, using Evans blue and iodinated plasma albumin, (8, 9, 15, 16) within one standard deviation. This would seem to indicate that all three methods measure the same protein pool.

From a practical standpoint, Cr^{51} has several advantages. Lipemia and hemolysis do not interfere with the determination. *In vitro* tagging of proteins is unnecessary, since 98 per cent or more of an injected dose of chromic chloride is immediately bound by the plasma proteins *in vivo*. The simple chemical solution of chromic chloride is stable for long periods. The low dose of radioactivity (0.1 rep.) permits repeated use of the Cr^{51} method on the same individual, and the amount of chromium (0.1 to 1.0 mg.) used in a single experiment is also sufficiently low to permit repeated administration with complete safety. Results of the determination are available within six to seven hours if a Geiger or proportional flow counter are used, for which the planchets must be dried. When the samples are counted wet in a gamma ray counter, the final results are available within one to two hours. This makes the technique a rapid and useful clinical tool. Furthermore this method makes possible the simultaneous determination of plasma volume and red cell mass with two forms of the same isotope, radioactive chromic chloride and sodium chromate.

SUMMARY

1. Radioactive chromic chloride with a half-life of 26.5 days has been used for the determination of plasma volume in man.

2. A saline solution of radioactive chromic chloride was injected into the circulation where it is immediately bound by the plasma proteins.

3. After a short period to allow for mixing, samples of blood were drawn at five-minute intervals and the radioactivity of the plasma was determined.

4. The circulating plasma volume was calculated by the isotope dilution principle, correcting for the loss of protein-bound radioactive chromic chloride from the circulation by a curve extrapolating the radioactivity to zero time.

5. The accuracy of the method was within 3 per cent as verified by hemorrhage and transfusion experiments with measured volumes of plasma.

6. The circulating plasma volumes of 21 normal adult males were determined:

Mean circulating plasma volume 2894 ml. ± 366
Mean plasma volume per Kg. body weight 39.3 ml. ± 4.9
Mean plasma volume per sq. m. surface area 1515 ml. ± 157

APPENDIX A

Assay of Cr^{51} and Dosage Determination

Cr^{51} is a soft x-ray emitter with a half life of 26.5 days. Recent determinations by A. W. Sunyar (17) have indicated that 10 per cent of the disintegrations are accompanied by gamma rays, in disagreement with a previous figure of 3 per cent given by Bradt and his co-workers (18). W. S. Lyon, at the Oak Ridge National Laboratory, has found 8.3 per cent gamma radiation in Cr^{51} (19).

We have standardized Cr^{51} against a Bureau of Standards radium standard, using a Lauritsen electroscope and a $\frac{1}{4}$ inch aluminum absorber. In the calculations, we used the revised Cr^{51} disintegration scheme with 10 per cent gamma radiation and made due allowance for the difference of the energy of the gamma rays of Cr^{51} and the energies of the several gamma rays of the radium standards. By this assay method we obtained results higher by a factor of 2.55 than by our earlier method (1). The errors involved in the several measurements of the present method, including the fraction of gamma rays per disintegration, indicate that the results are probably not accurate to better than 20 per cent.

We have measured samples that have also been assayed by Abbott Laboratories and agree with them to better than 20 per cent.

Using the Lauritsen electroscope assay, the counting efficiencies for Cr^{51} were determined on several kinds of counters (Table I). These efficiencies can only be considered as qualitative estimates, and in view of the complicated disintegration scheme should not be used to give the gamma ray or x-ray efficiency of any of the counters.

The radiation dosage has been recalculated on the basis of 10 per cent gamma radiation. Using the formula of Marinelli, Quimby, and Hine (4) we have:

$D = D_\beta + D_\gamma$

where D is total dosage and D_β and D_γ are the soft x-ray and gamma contributions, respectively.

$D_\beta = 88$ ETC rep.

where E = average energy per disintegration in MeV.; assuming a fluorescence yield of 0.263, this
$= 4.92 \times 10^{-3} \times 0.263$
$= 1.294 \times 10^{-3}$ MeV.

T = half life of the isotope, 26.5 days

C = concentration in microcuries per gram of tissue

$= 3.0$ C rep.

$D_\gamma = K_\gamma C\ g\ 10^{-1}$ rep.

where $K_\gamma = 1.44\ t\ I_\gamma\ 10^{-3}$

where t = half life of the isotope, 636 hours and $I_\gamma = 1.8$ r/mc.-hr.
$= 1.65$

g = geometrical area of distribution
$= 314 - 4140\,\mu$
where μ for .320 MeV. = .033
$= 177$

$= 29.2$ C rep.

It is assumed that the x-radiation affects the blood stream only, that the gamma rays irradiate the whole body, and that there is no excretion of Cr^{51}.[6] With these conservative assumptions: $D = D_\beta + D_\gamma$

If 100 μc of Cr^{51} are administered to an individual weighing 70 Kg.

$$D_\beta = \frac{3.0 \times 100}{5000} = .06 \text{ rep.}$$

and

$$D_\gamma = \frac{29.2 \times 100}{70 \times 1000} = .04 \text{ rep.,}$$

which represents the contribution arising from the gamma rays, neglected in the previous calculations.

$$D = .1 \text{ rep.}$$

Daily dosage = $D\lambda$ rep.

where λ = disintegration constant of Cr^{51} per day, 0.0261

= .003 rep. for 100 μc Cr^{51} in a 70 Kg. individual

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[6] The possibility of accumulation of Cr^{51} in the spleen as a result of red cell destruction at that site has also been considered. Data obtained on a series of dogs show a concentration of no more than 0.05 percent of the injected Cr^{51} dose per gram of spleen during the first three weeks after injection. The spleen in the dog therefore may receive twice the radiation that the blood stream does. However, since the disappearance curve of tagged red cells is steeper in the dog than in man, the human spleen may well receive a lower total dosage. Cr^{51} accumulation in the spleen following the injection of labeled erythrocytes does not appear to be significant in normal subjects; however, it may be considerably greater in patients with rapid red cell destruction from any cause.

APPENDIX B

Protocol and calculations of plasma volume determination

D. P., 21-year old male medical student; Wt., 68 Kg.; Ht., 178 cm.; Surface area, 1.83 sq.m.

1:30 p.m., 10 ml. of blood withdrawn from subject. Injected 32.5 ml. of $Cr^{51}Cl_3$ solution in saline through same needle in right antecubital vein.

Ten ml. samples of blood withdrawn from left antecubital vein at 1:35, 1:40, 1:45, and 1:50 p.m.

Sample	Geiger counts/ml.
Injected sol'n diluted 1:50	300 304
5 min. sample	150 149
10 min. sample	134 138
15 min. sample	128 142
20 min. sample	116 120

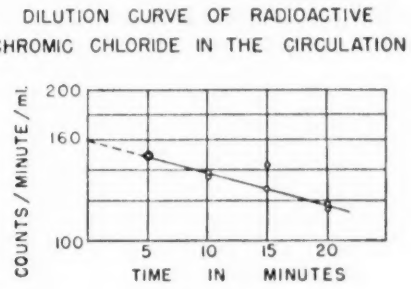

FIG. 1

Calculations: 32.5 ml. $Cr^{51}Cl_3$ solution injected × 302 counts/ml. × dilution of 50 =

	491,000	counts injected
−2%	9,820	counts lost to RBC's
	481,180	counts in plasma

$$\text{Plasma Volume} = \frac{481{,}180}{160 \text{ counts/ml. plasma at zero time}} = 3007 \text{ ml.}$$

APPENDIX C

Sample protocol and calculations of bleeding experiment

F. H., 32-year old normal male; Wt., 79 Kg.; Ht., 178 cm.; Surface area, 1.83 sq.m.

Initial Plasma Volume: 1:40 p.m., 10 ml. of blood withdrawn from subject.
Injected 28.8 ml. $Cr^{51}Cl_3$ solution in saline through same needle in right antecubital vein.
Ten ml. samples of blood withdrawn from left antecubital vein at 1:46, 1:51, 1:56, and 2:02 p.m.

Hemorrhage: 452 ml. blood removed. Hct. 47.0.

Final Plasma Volume: 2:22 p.m., 10 ml. control sample of blood withdrawn from subject.
Injected 28.8 ml. $Cr^{51}Cl_3$ solution in saline through same needle in right antecubital vein.
Ten ml. samples of blood withdrawn from left antecubital vein at 2:28, 2:37, and 2:47 p.m.

Sample	Geiger counts/ml.
Injected sol'n diluted 1:50	960 996
6 min. sample	393 379
11 min. sample	279 273
15 min. sample	224 223
22 min. sample	189 193
Control sample prior to 2nd injection	122 116
6 min. sample after 2nd injection	458 448
15 min. sample	311 317
25 min. sample	252 248

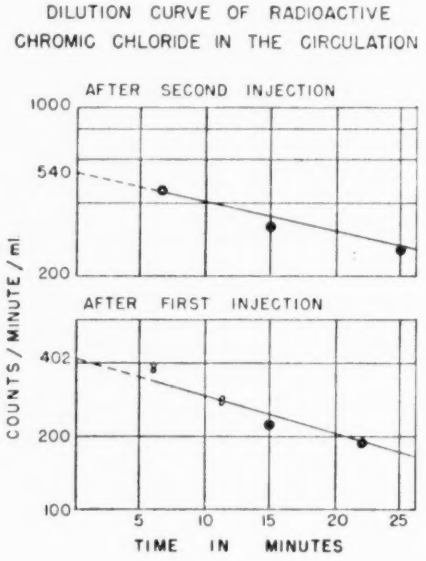

FIG. 2

Calculations: 28.8 ml. $Cr^{51}Cl_3$ solution × 978 counts/ml. × dilution of 50 =

	1,415,000	counts injected
−2%	28,300	lost to RBC's
	1,386,700	counts in plasma

$$\text{Initial Plasma Volume} = \frac{1{,}386{,}700}{402 \text{ counts/ml. plasma at zero time}} = 3449 \text{ ml.}$$

$$\text{Final Plasma Volume after Hemorrhage} = \frac{1{,}386{,}700 \text{ (2nd Injection)}}{540 \text{ counts/ml. plasma extrapolated to zero time} - 119 \text{ (counts remaining/ml. plasma prior to 2nd injection of chromic chloride)}} = 3294 \text{ ml.}$$

Initial Plasma Volume	3,449 ml.	
−Hemorrhage	240 ml.	
	3,209 ml.	Error = 2.5%

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THE SIMULTANEOUS DETERMINATION OF RED CELL MASS AND PLASMA VOLUME IN MAN WITH RADIOACTIVE SODIUM CHROMATE AND CHROMIC CHLORIDE [1]

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The use of radioactive chromium in the determination of the circulating red cell mass (1, 2) and the plasma volume (3, 4) in man has been reported previously. Anionic hexavalent chromium ($Na_2Cr^{51}O_4$) is readily taken up by erythrocytes *in vitro* and retained by them after reinjection without appreciable loss to the plasma for approximately 24 hours. Cationic trivalent chromium in the form of chromic chloride readily tags plasma proteins *in vivo* and *in vitro*. At least 98 per cent of the radioactive chromic chloride is bound to the plasma proteins with minimal loss of the isotope to the red cells.

Because of the selective affinity of erythrocytes for hexavalent anionic Cr^{51} and of the plasma proteins for trivalent cationic Cr^{51}, it seemed possible to use these two forms of the isotope for the simultaneous determination of red cell mass and plasma volume in human subjects.

Simultaneous measurements of red cell mass and plasma volume were made on ten normal adult subjects with radioactive chromium. The accuracy of the combined technique was confirmed by making determinations immediately before and after the transfusion of a measured volume of blood.

METHODS

I. Tagging, injecting, and sampling procedure

The combined method consists of tagging the subject's red cells *in vitro* with radioactive sodium chromate and reinjecting a saline suspension of the tagged cells. After the tagged cells have mixed in the circulation, the radioactivity of the subject's erythrocytes is determined and the circulating red cell mass may be calculated by the radioisotope dilution principle (1, 2). When the ten-minute sample of blood is withdrawn, a solution of radioactive chromic chloride is injected intravenously. The dilution curve of the isotope in the plasma is obtained by determining the radioactivity of four samples of plasma drawn at five-minute intervals after the injection of the chromic chloride, and the plasma volume may then be calculated by the radioisotope dilution principle (4).

Approximately 50 ml. of blood are drawn from the subject into a sterile heparinized syringe. This is poured into a sterile flask containing approximately 25 ml. of saline and 200 microcuries of Cr^{51} as $Na_2Cr^{51}O_4$.[2] The blood is allowed to stand at room temperature for one hour in contact with the isotope, with occasional shaking. At the end of this time the red cells have acquired about 50 per cent of the counts, or 100 microcuries Cr^{51}.

The contents of the flask are then filtered through sterile gauze and centrifuged in the cold. The supernatant saline and plasma are removed and the red cells are washed three times with cold sterile saline and finally resuspended in approximately 25 ml. of cold sterile saline.

An aliquot of this red cell suspension is saved for counting and the remainder, approximately 40 ml., is placed in a sterile calibrated syringe for reinjection. To reduce the chances of contamination and bacterial growth, the tagged red cells are reinjected the same day, and no febrile reactions have been encountered. Penicillin may be added to the injecting solution, as an additional precautionary measure.

Ten ml. of blood are drawn into an heparinized syringe from the antecubital vein, as the control sample for the plasma volume determination. Syringes are changed and the tagged red cell suspension is injected through the same needle. After ten minutes, another 10 ml. sample of blood is obtained and again syringes are changed for the injection of the chromic chloride solution (25 ml. of saline containing approximately 100 microcuries of Cr^{51}). After the injection of chromic chloride, four samples of blood are taken at five-minute intervals from the arm opposite to that used for injection.

II. Preparation of samples for counting

The radioactivity of the red cells and plasma can be determined in liquid samples on a liquid sample scintillation counter (W. S. McDonald Co., Inc., counter type 155, detector head type 152-H5) or on a well type gamma ray counter (Texas Co. type 18-20 TR). The red cells and plasma may also be dried, and counting can then be

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[2] $Na_2Cr^{51}O_4$ may be prepared from $Cr^{51}O_3$ by the method previously described (3) or may be obtained from Abbott Laboratories.

TABLE I

Counting rates of red cell and plasma samples on different counters

Type of counter	Per cent efficiency	Back-ground	Red cells		Plasma	
			Type of sample	Corrected counts per minute per sample	Type of sample	Corrected counts per minute per sample
Scintillation	2.96	450	*Liquid* 2 ml. 1:1 red cell hemolysate	2800	*Liquid* 2 ml. plasma	4430
Texaco Well	.3	390	6 ml. 1:1 red cell hemolysate	760	5 ml. plasma	1000
Geiger Muller Amperex Tube	.54	10	*Dried* 1 ml. 1:25 red cell hemolysate	20	*Dried* 1 ml. plasma	230
Geiger Muller Tracer Lab Tube	.78	18	1 ml. 1:25 red cell hemolysate	30	1 ml. plasma	335
Proportional flow	23.6	20	1 ml. 1:25 red cell hemolysate	200	1 ml. plasma	3350

done on an end-window Geiger counter (North American Phillips, tube type 62017-Amperex or Tracerlab Inc., tube TCG 3) or on a windowless flow type proportional counter (modification of the counter described by C. V. Robinson) (5).

A. Liquid sample counting method:

Four ml. of whole blood from the sample obtained 10 minutes after the injection of the tagged erythrocytes are placed in a 4 ml. hematocrit tube. This is centrifuged at approximately 2700 rpm. at a radius of 14 cm. for 60 minutes in the cold room. The supernatant plasma is removed and the remaining packed red cells are hemolyzed with exactly their own volume of distilled water. Two ml. aliquots of this hemolysate are pipetted into small rimless culture tubes for counting in the scintillation counter. The contents of each tube correspond to 1 ml. packed erythrocytes (Table I).

For the determination of the counts contained in the injected red cells, an aliquot of the tagged red cell suspension in saline is also centrifuged in a 4 ml. hematocrit tube for one hour at 2700 rpm. After removal of the supernatant saline, the red cells are hemolyzed in the same manner as the other sample, but the hemolysate is then further diluted with 25 volumes of water in order to bring its counting range within that of the other samples. Hematocrit corrections are not necessary since the amount of plasma or saline trapped is constant and both plasma and saline are free of counts. Washing of red cells to remove plasma, particularly in samples obtained after the injection of radioactive chromic chloride, does not seem to be necessary.

For the plasma volume determination, the blood sample obtained ten minutes after injection of the tagged red cells and just before the injection of the chromic chloride solution serves as the control sample. This and the four samples obtained after the injection of chromic chloride are centrifuged to separate the plasma. Exactly 2 ml. aliquots of these five plasma samples are pipetted into small rimless culture tubes for counting. A saved aliquot of the injected chromic chloride solution is diluted 1:50 and 1 ml. of this is also counted in a small rimless culture tube.

All of these specimens are then ready for immediate counting in the scintillation counter. For a counter with lower efficiency, such as a well type gamma ray counter, larger aliquots of red cell hemolysates and plasma (5 to 7 ml.) should be used in order to obtain reasonable counting rates (Table I). No correction for absorption is necessary for either of these gamma ray counters.

B. Dried sample counting method:

The red blood cells can be prepared for counting as dried ground cells or as dried 1:25 red cell hemolysates (2). The hemolysate method is simpler and more rapid than the ground red cell method and has been used exclusively in this study. In preparing the hemolysates, 4 ml. hematocrit tubes are filled approximately one-third with whole blood and centrifuged at 2700 rpm. for one hour. After removal of the plasma, the packed red cells are diluted to precisely five times their initial volume with distilled water as read from the calibration on the hematocrit tubes. The tubes are covered with Parafilm and inverted at least ten times to ensure complete hemolysis.

Two-tenths ml. aliquots of the hemolysates are pipetted into aluminum planchets. Eight-tenths ml. of distilled water are added to the planchets immediately, and the contents of each planchet are carefully mixed by stirring with a clean wire. The hemolysates are dried in air overnight or in an oven at 60° C. for about five hours and then stored in a desiccator before counting (Table I).

The red cells obtained ten minutes after the injection of the tagged erythrocytes, as well as an aliquot of the original saline suspension of tagged cells itself, are treated identically. The absorption correction is, therefore, considered uniform and may be omitted from the calculations.

For the plasma volume determination, all blood samples are spun in the centrifuge to separate the plasma. Duplicate planchets containing one ml. of plasma each are prepared from the samples obtained just before and during the 20 minutes after the injection of the chromic chloride solution. An aliquot of the injected chromic chloride solution is diluted 1:50 with water and ½ ml. of this diluted solution is overlaid with 1 ml. of the subject's control plasma obtained at the beginning of the experiment and free of counts. The plasma and solution are well mixed in the planchets with a clean wire. Since all planchets contain 1 ml. of plasma, the absorption correction may be considered uniform and need not enter into the calculations. If the control plasma contains counts from a previous injection of isotopes, any normal plasma, free of counts, may be used, provided that the protein concentration in both plasmas is approximately the same.

All samples are dried over-night in air or in an oven at 60° C. for about five hours. They are then stored in a desiccator until they are counted on an end-window Geiger counter or a windowless flow type proportional counter (Table I).

The plasma sample obtained just before the injection of the chromic chloride solution serves as a control to assure that no free counts remain in the plasma from the previous injection of tagged erythrocytes.

III. Calculations and dosage

The circulating red cell volume of the subject is given by the formula:

$$\text{Red cell volume in ml.} = \frac{\text{Total counts in tagged erythrocytes injected}}{\text{Counts per ml. packed red cells in the subject's circulation}}$$

The plasma volume is calculated by the formula:

$$\text{Plasma volume in ml.} = \frac{\text{98 per cent of counts injected as chromic chloride}}{\text{Counts per ml. of plasma at zero time}}$$

As previously given, the total radiation dose for 100 microcuries of Cr^{51} corresponds to 0.1 rep. In the simultaneous determination of red cell and plasma volume, the subject receives approximately 200 microcuries Cr^{51}, or a total radiation dose of 0.2 rep. This is within the tolerance limits for human use, as is the total dose of chromium of 0.2 to 2.0 mg.

RESULTS

Combined determinations of circulating red cell mass and plasma volume have been done on ten normal individuals (Table II). The mean circulating red cell mass of these ten normal adults was 2081 ml., compared to a mean value of 2351 ml., obtained on 25 normal male students in whom the red cell mass alone was determined. The mean plasma volume in these same individuals was 2795 ml., compared to a mean value of 2894 ml., obtained on 21 normal adult males in whom the plasma volume alone was determined. Calculated on the basis of body weight, the combined method yielded a mean red cell mass of 30.3 ml. per Kg. and a mean

TABLE II

Red cell mass and plasma volumes in ten normal adults

Subject	Total plasma volume *ml.*	Plasma volume *ml./Kg.*	Total red cell mass *ml.*	Red cell mass *ml./Kg.*	Venous hct.	Body hct.	Body hct. / Ven. hct.
Le	2180	37.8	1420	24.6	44.0	39.4	0.90
Do	2766	40.5	1909	27.9	48.8	40.8	0.84
Da	2803	41.6	2835	42.1	50.6	50.1	0.99
Ki	2440	31.6	2550	33.0	48.0	51.1	1.07
Mu	3130	42.3	2390	32.3	43.1	43.2	1.01
Ga	2110	34.7	1834	28.8	50.5	46.5	0.92
Ph	3770	48.5	2770	35.8	44.5	42.1	0.96
Ma	3015	47.7	1680	26.6	49.4	35.8	0.73
Jo	3005	41.5	1700	23.7	45.2	36.0	0.80
Si	2730	45.0	1720	28.4	41.2	38.1	0.93
Average	2795	41.1	2081	30.3			0.91
Standard deviation	±498	±5.6	±508	±5.6			±0.10
Standard error of mean	157	1.7	161	1.7			0.03

plasma volume of 41.1 ml. per Kg. The mean values, when the determinations were done separately on normal adult males, were 31.8 ml. red cells per Kg. and 39.3 ml. plasma per Kg. The body hematocrit was calculated by the formula: $\frac{\text{Red cell mass}}{\text{Red cell mass} + \text{Plasma volume}}$, and the ratio of the body hematocrit to the venous hematocrit was found to be 0.91 ± 0.10 (Table II). This is the same value as that obtained by Gibson, Peacock, Seligman, and Sack, using radioactive iron and Evans blue dye (6).

Comparison of the values obtained by different counting methods revealed agreement within 2.3 per cent, which compares well with the limits of accuracy of the method as a whole (Table III).

TABLE III

Reproducibility of a red cell mass and plasma volume determination on different counters

	Scintillation counter *ml.*	Geiger counter *ml.*	Flow counter *ml.*	Mean error %
Plasma volume	2730	2705	2730	0.5
Red cell volume	1720	1650	1660	2.3

What differences do occur are both positive and negative and presumably are not due to any systematic discrepancies between counting procedures.

In one individual, plasma and red cell volume determinations were performed immediately before and after transfusion of a measured quantity of whole blood (Table IV). The results agreed with the calculated values within 3 per cent, the limits of accuracy obtained in the separate determinations of red cell and plasma volume.

DISCUSSION

The feasibility of performing both red cell and plasma volume determinations by means of a single isotope, Cr^{51}, is of considerable interest. It has been shown by various investigators (6, 7) that the venous hematocrit does not represent the body hematocrit and that estimations of blood volume based on either plasma or red cell determinations and the venous hematocrit will necessarily be subject to considerable error. Various techniques have therefore been tried to determine red cell and plasma volume simultaneously (6–10).

From a practical standpoint, the Cr^{51} method has several advantages over these other combined methods. It requires minimum counting equipment and standardization. Further, it has now been shown that when the two procedures are done simultaneously, the technique remains simple and accurate. With the perfection of more efficient liquid sample gamma ray counters, results of red cell and plasma volume determinations can be obtained within one to two hours. This makes the method practicable for all types of clinical studies. The values obtained by counting samples in the liquid and dried state do not differ significantly.

If the subject has received a previous injection of a radioisotope, and counts remain in the plasma prior to the plasma volume determinations, the counts present in the plasma prior to the injection of chromic chloride must be corrected for in the calculation of the plasma volume.

It has been shown in the previous communication that less than 2 per cent of the intravenously injected chromic chloride tags the red cells. This was further demonstrated by determining the radioactivity of samples of red cells obtained at varying intervals before and after the injection of chromic chloride and calculating the red cell mass. The counts per ml. of packed erythrocytes and consequently the circulating red cell mass remained unchanged throughout the experiment. Washing of the packed red cells prior to counting was not necessary, since the washed and unwashed

TABLE IV

Transfusion experiment

H. D. Age 66, Wt. 60 Kg.

	Initial volume by Cr^{51} method *ml.*	Measured volume transfused *ml.*	Calculated final volume *ml.*	Final volume by Cr^{51} method *ml.*	Per cent error %
Red cells	2005	241	2246	2240	0.3
Plasma	2180	259	2439	2450	0.5

samples had essentially the same radioactivity. With the use of the four ml. hematocrit tubes and one hour of centrifugation at 2700 rpm., the amount of trapped plasma did not alter the accuracy of the results.

Conversely, radioactive sodium chromate is so firmly bound to the red cells, that essentially no counts were found in the plasma ten minutes after the tagged red cells had been injected. Consequently, radioactive sodium chromate did not interfere with the plasma volume determination. It has previously been shown that the counts in the plasma are negligible for 24 hours after the injection of erythrocytes tagged with radioactive sodium chromate (*2*).

SUMMARY

1. Radioactive sodium chromate and chromic chloride have been employed in the simultaneous determination of both red cell mass and plasma volume in man.

2. A small aliquot of the subject's erythrocytes was tagged rapidly *in vitro* with radioactive sodium chromate and reinjected within a few hours.

3. A saline solution of radioactive chromic chloride was injected into the circulation, where it is rapidly bound by the plasma proteins.

4. The radioactivity of the subject's erythrocytes and plasma was determined after the injected tracer doses had mixed in the circulation.

5. Both red cell and plasma volume were calculated by the isotope dilution principle.

6. The specific affinity of chromic chloride for the plasma proteins is such that less than 2 per cent of the injected material is lost to the red cells.

7. The bond of sodium chromate to the erythrocytes is sufficiently strong so that there is no significant loss of radioactivity to the plasma.

8. The two forms of the isotope could therefore be present in the circulation simultaneously without interfering with the accuracy of either the red cell or the plasma volume determination.

9. The mean values for red cell mass and plasma volume obtained by the combined method agree favorably with those obtained when radioactive sodium chromate and chromic chloride are administered separately.

ACKNOWLEDGMENTS

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STORAGE OF BLOOD BELOW 0° C. IN LIQUID STATE[1]

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INTRODUCTION

In their original observations in 1916, Rous and Turner (1) recognized the importance of refrigeration in the preservation of blood. In recent years Parpart and his co-workers (2) studied the effect of different temperatures. The temperatures most suitable for survival of human red cells were found to lie between 4° C. and 9° C. with an optimum at about 7° C. This has been confirmed by the *in vivo* studies of Gibson and his co-workers (3). Rapoport (4) studied the survival rate of red cells during storage at 4° C. and 25° C. and found that storage for two days at 25° C. produced changes equivalent to those of two weeks storage at 4° C. Temperatures above 10° C. favor more rapid deterioration of red cells. Storage temperatures below 0° C. could not be evaluated because blood freezes and then hemolyzes completely upon melting.

Such a study is possible only if blood can be kept liquid below 0° C. by some physical or chemical means, and the procedure itself is not deleterious to red cells. The present investigation attempts this by the use of high pressures or of alcohol to depress the freezing point of the whole blood.

Water can be prevented from freezing by application of hydraulic pressure. The phenomenon is valid with 2,045 atmospheres to − 22° C. For greater pressures the freezing points are not lowered as the water-ice phase diagram indicates. Bridgman (5) studied the phase changes for the full range of 10,000 atmospheres (Figure 1). At higher pressures than 2,045 atmospheres water freezes at increasingly higher temperatures. In the present investigation pressure was kept below 2,045 atmospheres. Thus a pressure of 590 atm. will lower the freezing point of water to − 5° C.; 1,090 atm. to − 10° C.; 1,540 atm. to − 15° C.; and 1910 atm. to − 20° C.

The effect of pressure on red cells was studied by Callery and Portier (6) as early as 1910. It was found by these authors that 10 atm. pressure did not cause hemolysis and that hemolysis occurs when the pressure is greater than 300 atm. Fontaine (7) found that defibrinated blood, kept for seven hours at 500 atm. pressure, showed only slight hemolysis. Red cells suspended in saline hemolyzed more rapidly. Haubrich (8) and Ebecke (9) found that pressure changed the physical form of the red cells, giving them a more spherical shape. Spherocytes are considered more susceptible to hemolysis. These early investigations indicated that pressure is by no means innocuous to red cells.

The storage of blood under pressure was considered worthy of further investigation because earlier studies were limited to only a few hours of storage of defibrinated blood or red cells suspended in saline, and were all carried out at room temperature. Since this study was completed, our attention has been called to the favorable report by Jessiman and Walter (10) using 22,500 lbs. per sq. inch and low temperatures.

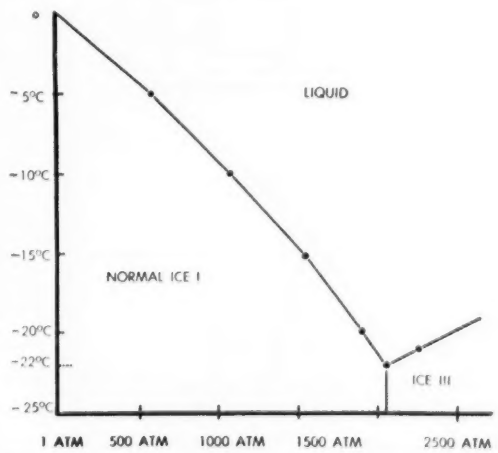

FIG. 1. WATER-ICE PHASE DIAGRAM AT TEMPERATURES AND PRESSURES CORRESPONDING TO THIS INVESTIGATION

[1] This work was made possible by a research grant from the Public Health Service.

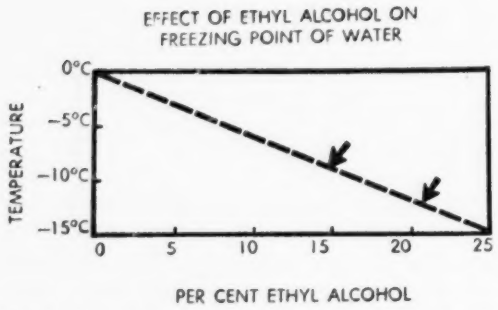

FIG. 2. EFFECT OF ETHYL ALCOHOL IN FREEZING POINT OF WATER

Alcohol lowers the freezing point of water, and binary mixtures freeze at temperatures between 0° and − 115° C. depending on composition. Figure 2 shows the depression of freezing point of water by the addition of alcohol in concentrations corresponding to the ranges in this investigation. The effect on the depression of the freezing point by an equivalent amount of alcohol is greater in blood, due to the presence of salts and proteins. The toxicity of alcohol is low. Up to 100 ml. of alcohol have been given intravenously daily. Alcohol can be removed quite readily from blood.

Alcohol is hemolytic to red cells, and its use to lower the freezing point of blood has, thus far, not been considered. Alcohol diluted with distilled water and added to blood causes a prompt and complete hemolysis. Alcohol is freely permeable through the red cell membrane, and isotonic saline is necessary for osmotic equilibrium. If osmotic hemolysis is prevented, alcohol hemolysis is only moderate at room temperature and is much retarded at 4° C. Below 4° C., alcohol is not hemolytic in a wide range of concentrations, providing isotonicity is maintained.

In view of this observation, it was decided to study the storage stability of whole blood containing various concentrations of alcohol. It was found practical to limit the study to the storage of blood at − 7.5° C. and − 12.5° C. by the addition of 15 per cent and 21 per cent alcohol, respectively.

MATERIAL AND METHODS

Whole blood was obtained from the Blood Bank of the Queens General Hospital in standard A-C-D solution. The A-C-D solution contained 1.33 per cent sodium citrate, 0.4 per cent citric acid, and 3 per cent glucose. Twenty-five cc. of such A-C-D solution were added to 100 cc. of whole blood. Two commercial low temperature storage cabinets were converted to constant temperature units operating in any pre-set range between 0° to − 25° C. by the use of a fan within the cabinet and adequate controls.

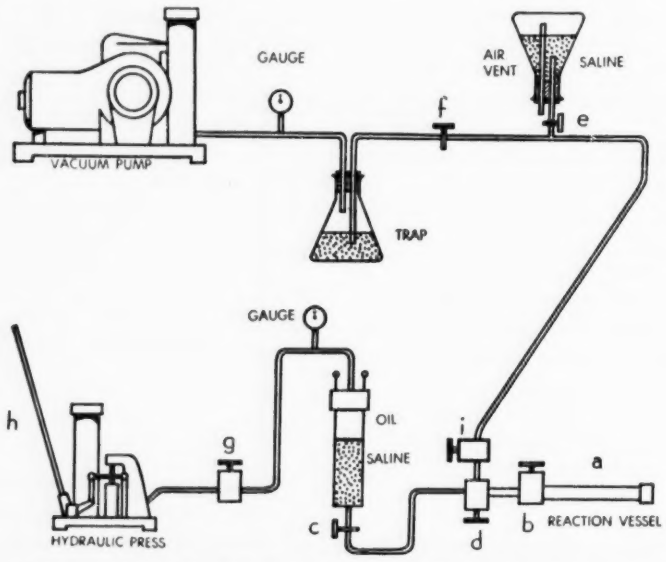

FIG. 3. SYSTEM FOR APPLICATION AND RELEASE OF HIGH PRESSURE

The initial study aimed to determine the effect of high pressure (Figure 3). The procedure for applying the pressure was as follows: 15 ml. of blood were introduced to fill completely the high pressure reaction vessel (a). The vessel was closed by valve (b) on the reaction vessel, and valve (b) was then attached to pressure system. To evacuate tubing leading from hydraulic press to the reaction vessel (a), valves (d, i, f) were opened and system evacuated by vacuum pump. Following evacuation, valve (f) was closed, valve (e) opened to allow saline solution to fill tubing. Now for pressure application, valves (e, i) were closed and, to prevent reflux, a slight pressure was applied to the system by manually pumping handle (h) with valve (b) still closed and valve (c) opened.

Now the valve (b) was opened and the desired pressure applied to the blood in reaction vessel (a). Valve (b) was closed and the pressure vessel was removed for storage.

To release pressure in the vessel, it was first warmed to 4° C. then reconnected to the system filled with saline. With valve (c) closed, the tubing was filled with saline. Now valve (i) was closed, and valves (c, d) were opened and a pressure applied to the system which was equal to that in the container. Valve (b) was opened and the pressure was matched. The pressure was then released rapidly or slowly, as desired, through valve (g). This ordinarily was accomplished in a few minutes.

For a study of the effects of alcohol, a commercial 95 per cent preparation was diluted by saline in a proportion of 65 parts alcohol to 35 parts of physiological saline. Larger amounts of alcohol precipitate blood proteins. Alcohol at 21 per cent concentration was the maximum used because hemolysis occurred at − 12° C. with higher levels. Dextrose was added to give a 500 mg. per cent concentration. The addition of dextrose to the alcohol seemed to prevent hemolysis during storage. One part of this (alcohol, dextrose, saline) or A-D-S solution containing 65 cc. of 95 per cent alcohol, 35 cc. of saline, and 500 mg. of glucose was added to two parts of whole A-C-D blood to obtain an approximate 21 per cent alcohol concentration. Also, one part of another A-D-S solution containing 42 parts of 95 per cent alcohol, 58 cc. of saline and 500 mg. of glucose, was added to one part of A-C-D blood to produce a similar approximate 21 per cent alcohol concentration in a more diluted blood. As alcohol was found hemolytic to red cells at room temperature, the A-D-S solution and all the syringes and containers used were cooled to − 12° C. In some samples only half of the calculated amount of A-D-S solution was added to the A-C-D blood at first and the diluted blood was stored for one and one-half hours at − 12° C. before the second half of the A-D-S solution was added.

The osmotic resistance of the red blood cells to 0.6 per cent saline was determined by the method of Parpart (11) and was expressed in per cent of total red cells hemolyzed. Samples taken from cold storage were tested without delay because blood diluted with alcohol hemolyzes rapidly at room temperature.

For each sample the degree of hemolysis was determined by the ratio of the hemoglobin content of the supernatant after centrifuging for five minutes at 1500 r.p.m. over the hemoglobin content of the whole blood, and is referred to as *percentage hemolysis*. The hemoglobin was determined by the standard HCl method in a Fischer Electrohemometer.

RESULTS

Pressure

Blood undergoes complete hemolysis at atmospheric pressure if the temperature is lowered from 4° C. to − 8° C., while blood under 680 atmospheres pressure shows about the same degree of hemolysis at 4° C. as at − 8° C. with percentage hemolysis of 54 and 59, respectively, after 14 days of storage. It can be assumed that freezing was prevented by the application of pressure. It is

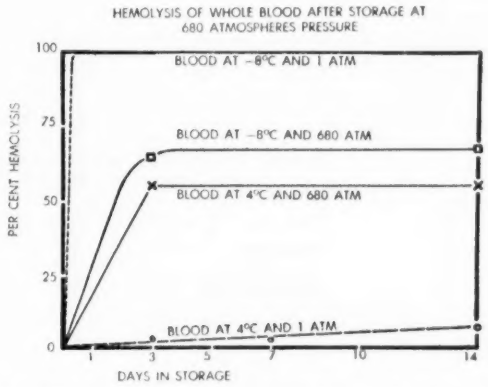

FIG. 4. HEMOLYSIS OF WHOLE BLOOD AFTER STORAGE AT 680 ATMOSPHERES PRESSURE

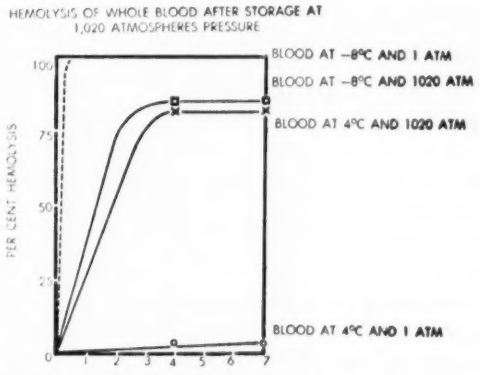

FIG. 5. HEMOLYSIS OF WHOLE BLOOD AFTER STORAGE AT 1020 ATMOSPHERES PRESSURE

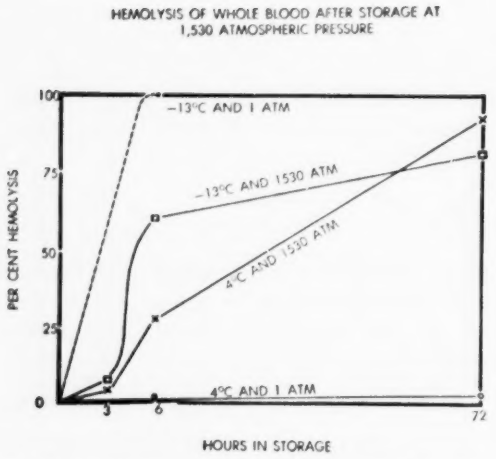

FIG. 6. HEMOLYSIS OF WHOLE BLOOD AFTER STORAGE AT 1530 ATMOSPHERES PRESSURE

also evident that blood under pressure develops considerable hemolysis, irrespective of temperature. It appears that pressure accelerates the reactions responsible for the breakdown of blood in storage. The harmful effects of pressure were found to increase with the amount of pressure applied and of the duration of its application. Figures 4, 5, and 6 illustrate these effects for 680, 1,000, and 1,530 atmospheres of pressure. Figure 4 represents the lowest pressure at which a significant lowering of the freezing point of blood could be obtained, and Figure 6, the pressure corresponding to the maximum lowering of the freezing-point attainable on the basis of the ice-water phase diagram. It is interesting to note that the harmful effects of pressure are not instantaneous but develop with time in storage, the hemolysis increasing with higher pressures (Table I).

Clotting of blood in A-C-D solution, which occurred in storage under pressure, merits further study. It may be the result of a physico-chemical alteration of ionic equilibrium of the blood in the anticoagulant mixture.

Table I

Percent Hemolysis*of Whole Blood Stored at Various Pressures and Temperatures and Duration of Storage

Treatment	Duration of Storage		
	3 Hrs.	6 Hrs.	72 Hrs.
1530 atm. and 4°C	3.5	26	91 89 74
1530 atm. and -13°C	6.7	58	83
1 atm. and 4°C	—	0.8	1.1
1 atm. and -13°C		98	100
	4 days	7 days	
1020 atm. and 4°C	75 89 71	84 80 81	
1020 atm. and -8°C	79	92	
1 atm. and 4°C	4.5	2.0	
1 atm. and -8°C	100	100	
	3 days	7 days	14 days
680 atm. and 4°C	54 51 76	—	58 49 59
680 atm. and -8°C	72		59
1 atm. and 4°C	4.7	2.5	5.1
1 atm. and -8°C	100	100	98

* Defined as hemoglobin in supernatant after 1500 r.p.m. for 5 min. over hemoglobin of whole blood.

Alcohol

Figure 7 illustrates the grade of spontaneous hemolysis which occurs in A-C-D blood stored at − 12° C. in A-D-S solution as compared with blood stored at 4° C. in A-C-D solution alone. An increase of the spontaneous hemolysis occurred during storage in both at about the same rate.

Figure 8 illustrates the degree of osmotic resistance found in 0.6 per cent solution of sodium chloride, of blood stored at − 12° C. in A-D-S solution as compared with the same stored at 4° C. to which A-C-D solution was added. The difference is significant. A decrease of the resistance of the red blood cells is manifest in both during storage, less marked in the blood stored at − 12° C., provided that blood is not diluted to less than 54 per cent of its original volume. If blood is diluted to 27 per cent or 40 per cent of its original volume, the osmotic resistance is greatly diminished. Blood collected in a blood bank is already diluted to 80 per cent by the addition of A-C-D solution. Using one part of A-D-S solution to one part of A-C-D blood brings the blood volume to 40 per cent. By using two parts of A-C-D blood to one part of A-D-S solution, the blood volume is 54 per cent and by using two parts of A-D-S solution to one part of A-C-D blood, the volume drops to 27 per cent. Parpart (11) found a rapid decrease of osmotic resistance in blood stored at 4° C. if the volume of blood in the final diluent dropped below 40 per cent.

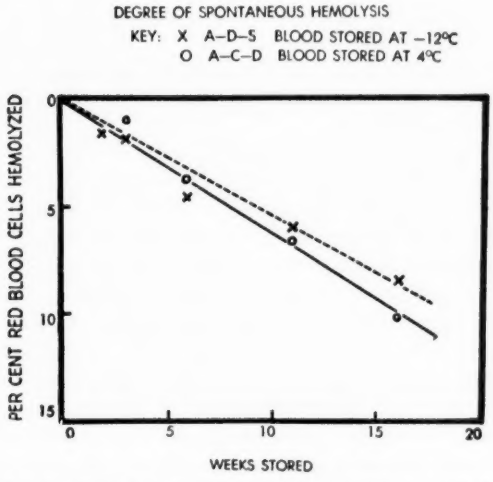

FIG. 7. DEGREE OF SPONTANEOUS HEMOLYSIS AT − 12° C. IN ALCOHOL-DEXTROSE SALINE

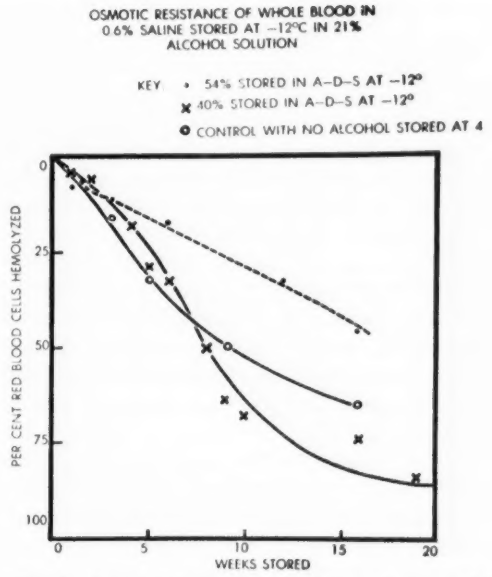

FIG. 8. OSMOTIC RESISTANCE OF WHOLE BLOOD TO 0.6 PER CENT NaCl STORED AT − 12° C. IN 21 PER CENT ALCOHOL-DEXTROSE SALINE

DISCUSSION

Attempts were made to lessen the hemolytic effect of pressure, by control of several variables. Rapid application and rapid release of pressure (1,500 atm.) to a sample of blood caused some immediate hemolysis, but the amount of damage was not considerable, being 0.28 Gm. hemoglobin per 100 ml. in the treated sample, as compared with 0.18 Gm. in the control, measured in the plasma. Blood held under 340 atmospheres for seven days showed no hemolysis, while another sample held under 680 atmospheres for the same period presented considerable hemolysis. A third sample had 340 atmospheres pressure applied for four days, and then an additional 340 atmospheres for three more days. The sample was as hemolyzed as if 680 atmospheres had been applied at once. The slow application and the slow release of pressure (up to 1,800 atm.) was tried at the rate of 10 atmospheres per minute. Again the harmful effects were of the same general order of magnitude

at the different pressures in storage as that found in the controls with single pressure applications.

The question whether the presence of air under pressure affected the red cells was considered. Pressure of 1,500 atmospheres was applied to two samples of blood, and air was not evacuated in one of them. The pressure was maintained for thirty minutes. In the control sample where the vacuum pump emptied the reaction vessel and pressure system as usual, the hemoglobin value of the overlying plasma was 0.09 Gm. hemoglobin per 100 ml. The test sample, without preliminary evacuation of the system before pressure application, had a hemoglobin value in the plasma of 0.11 Gm. The presence of air in the system did not increase hemolysis.

The application of silicone coating to the walls of the high-pressure reaction vessel did not reduce the extent of hemolysis. Samples of blood stored seven days at 4° C. under 340 atmospheres pressure showed a 0.3 Gm. hemoglobin in the plasma of the uncoated container and 0.5 Gm. hemoglobin in the blood sample in the silicone coated container.

Changes in the acidity, alkalinity, and osmotic pressure were studied. The addition of $NaHCO_3$ in amounts of 0.2 ml. normal $NaHCO_3$ to 20 ml. blood, prior to the application of pressure, did not prevent hemolysis. Adding HCl in amounts of 0.2 ml. normal HCl solution per 20 ml. blood was also tested. Treated samples were stored at − 7.5° C. under 545 atmospheres pressure. When tested, the sample treated with HCl contained 4.1 Gm. per 100 ml. and the control 3.7 Gm. per 100 ml. hemoglobin in the plasma. The addition of identical acid and alkaline solutions to blood without pressure did not show equivalent hemolysis during identical periods. The addition of distilled water in amounts of 10 per cent of original volume to one sample, and the addition of 0.2 Gm. NaCl to 20 ml. of blood, did not reduce but increased the degree of hemolysis at 545 atmospheres pressure.

The effects of instantaneous or rapid freezing and melting were studied. Samples of blood were subjected to 1,020 atmospheres pressure and cooled to − 11° C. The pressure was released suddenly so that instantaneous freezing would occur. After storage, the samples were defrosted by initially applying 1,020 atmospheres pressure instantaneously and then warming to 4° C. This did not prevent cell rupture, and complete hemolysis resulted.

The effect of pressure on red cell mass without plasma was found to be the same as on whole blood.

As noted, blood in A-C-D solution, if held under 1,020 to 1,530 atmospheres pressure for a few days, showed complete clotting. The addition of heparin or sodium oxalate to the A-C-D blood prevented clotting caused by such pressure. Since sodium oxalate prevented such clotting it may be that the original clotting was related to some shift in the ionization of calcium under pressure.

The rate of application and release of pressure, presence or absence of air under pressure, silicone coating of reaction vessel, addition of acid or alkali, change in osmotic pressure, and rapid freezing and melting were investigated. Varying each of the factors did not diminish the hemolysis.

The damage to the red cell caused by pressure may result from an altered metabolism of the cell. In most physical and chemical reactions, volume change occurs. Pressure opposes any change that increases volume and promotes a change that decreases volume. It is reasonable to assume that during storage under high pressures some metabolic reactions are promoted while others are retarded, depending on the volume changes in the individual chemical reactions. The sum of the effects of pressure on the red cell seems to favor hemolysis. Generally, cold retards metabolic activities of cells, and it was hoped that any damage caused by pressure would be compensated for by low temperature storage at − 13° C. It was disappointing to find that considerable hemolysis occured under pressure regardless of the temperature investigated.

In the experiments described, whole blood was successfuly stored at − 13° C. in the liquid state under pressure. Considerable change occurred in the blood with the increased pressure at any temperature investigated, as evidenced by the hemolysis, the extent of damage being related to the amount, and duration of pressure applied. Deterioration increased during storage. This paralleled the results of earlier investigations at room temperature on defibrinated blood or red cells suspended in saline. Our results do not parallel the recent favorable report of Jessiman and Walter (10) who subjected blood to 22,500 lbs. pressure per square inch and preserved it at − 15° C.

A few observations were made on animals. From each of five rabbits 20 ml. of blood were collected in 4 ml. A-C-D solution and cooled to 4° C. in a refrigerator. One part of blood was diluted by one part A-D-S solution containing 42 per cent alcohol, and the samples were stored at − 12° C. for four months. Following four months storage the supernatant fluid of the sample was syphoned off, replaced by saline and reinjected into the same rabbit. Unfortunately three rabbits died immediately following reinjection. The presence of fibrin clots accumulated during the long storage may have been instrumental in these deaths. Blood of the last two rabbits was filtered through cheese cloth and the rabbits survived reinjection well. The grade of spontaneous hemolysis of the blood, reinjected to one rabbit which survived was 6 per cent and the test of osmotic resistance against 0.6 per cent sodium chloride solution showed 78 per cent hemolysis.

Although blood stored at − 12° C. in the liquid state in alcohol and tested for spontaneous hemolysis and osmotic resistance did not show a striking improvement as compared with blood stored at 4° C., the possibility still exists that the addition of alcohol may be developed for long range preservation of blood. Storage of blood is affected by many factors, and only a few were evaluated. The evaluation test procedures performed dealt with the structural and osmotic state of the cells only. These are of primary interest but many other changes, which are related to metabolic activities of the cells, may be directly affected by low temperature. These changes are not revealed by the tests used. The rate of enzyme deterioration, glycolysis, lactic acid formation, changes of pH and potassium content of red cells were all found much reduced at 4° C. Below 0° C. such studies are not possible until the problem of prevention of hemolysis by freezing is solved. Such studies could not be undertaken in the present investigation. Determinations of spontaneous hemolysis and osmotic resistance of red cells do not give information concerning more subtle changes. Ability of the red cells to survive blood transfusion *in vivo* (as determined by the radioactive tagging with phosphorus, iron, etc.), is a more acceptable criterion of the value of preservation of blood below 0° C. in liquid state in A-D-S solution.

SUMMARY AND CONCLUSIONS

1. The preservation of blood at temperatures below freezing, kept liquid by the use of high pressures and by ethyl alcohol, was investigated.

2. The damage caused by pressure in the range investigated was considerable. High pressure low temperature preservation of whole blood does not seem to be a promising procedure for long term mass preservation of whole blood.

3. The presence of 21 per cent ethyl alcohol in the blood permits storage of whole blood at − 12° C. in liquid state. At this temperature 21 per cent alcohol does not hemolyze red cells.

4. The spontaneous hemolysis in blood, kept from freezing by alcohol and dextrose after four months storage at − 12° C., was equal to, or slightly less than, in blood stored at 4° C. in A-C-D solution alone.

5. Osmotic resistance of the red cells showed a definite but not remarkable improvement in the blood stored at − 12° C. in alcohol.

6. It is urged that the use of alcohol for the low temperature long range storage of blood and other tissues be studied.

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ALTERATIONS OF FLUID AND ELECTROLYTE DISTRIBUTION AND RENAL FUNCTION IN DIARRHEA OF INFANCY[1]

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INTRODUCTION

The salt and water lost during acute diarrhea was originally presumed to derive almost entirely from the extracellular compartment (1, 2). Subsequently, it was realized that a significant loss of potassium occurred, in excess of that which might be attributed to the negative nitrogen balance (3, 4, 5). This observation, with the assumption that transfer of body water depends upon primary transfer of base, led to the suggestion that considerable depletion of intracellular fluid also occurred (5, 6). Results obtained by balance studies, interpreted on the premise that the distribution of chloride is limited to the extracellular fluid, supported the belief that diarrhea of infancy induces primary depletion of extracellular water and salt with an additional loss of intracellular water and potassium (7, 8). These data also suggested that the early period of treatment may be characterized by a further depletion of the intracellular fluid volume, coexistent with an overexpansion of the extracellular compartment (7, 8).

The recent development of methods for the estimation of the fluid phase of the extracellular compartment with inulin, sucrose, and ferrocyanide, and of total body water with deuterium oxide and antipyrene suggested the use of these substances to estimate directly alterations in body water in diarrhea of infancy. Accordingly, serial determinations of plasma volume (T-1824 space), extracellular volume (inulin space), total body water (deuterium space), and renal function (inulin and p-aminohippurate clearances) were made during the acute stage of dehydration and during the subsequent period of treatment and recovery.

METHODS

Infants from two to eight months of age suffering from acute diarrhea were selected for study if clinical signs of dehydration (sunken fontanelle, soft eyeballs, dry mouth, decreased skin turgor) were present and if treatment could be safely postponed for six to eight hours. The volumes of distribution of T-1824, inulin and heavy water, glomerular filtration rate and renal plasma flow, hematocrit, plasma protein concentration and plasma electrolyte concentrations were measured prior to the institution of therapy.

These studies were repeated serially at two to seven day intervals during treatment. Following the initial studies, treatment was begun with 0.45 gram per cent solution of sodium chloride by continuous venoclysis at the rate of 150 to 200 ml. per kilogram of body weight per day. Appropriate quantities (75 to 200 ml.) of 1/6 molar lactate were administered to correct metabolic acidosis. Some of the more severely ill infants received in addition 10 to 20 ml. per kilogram of blood or plasma.

After signs of dehydration had disappeared (usually at the end of the first 24 hours of treatment) intravenous infusions of 0.45 to 0.225 gram per cent solution of sodium chloride were continued in decreasing quantities until oral intake was considered adequate. After 12 to 24 hours of starvation the infants were fed dilute evaporated milk formulae. Thereafter, the volume of the feedings and the proportion of evaporated milk were gradually increased according to individual tolerance. The length of time during which parenteral therapy was continued varied from infant to infant ranging between two to seven days. Two of the infants received 6 mM per kilogram per day of potassium chloride by mouth. Normal infants in the same age range, free of infection and metabolic disease on standard evaporated milk formulae, served as controls.

The volume of distribution of inulin was measured by the constant infusion technique, in which the amount of inulin excreted after the cessation of an equilibrating infusion is divided by the equilibrium plasma level (9). The infusion time necessary for the attainment of equilibrium distribution of inulin in the normal infant was estimated in two ways: *1)* repeat determinations of the volume of distribution of inulin were made in the same

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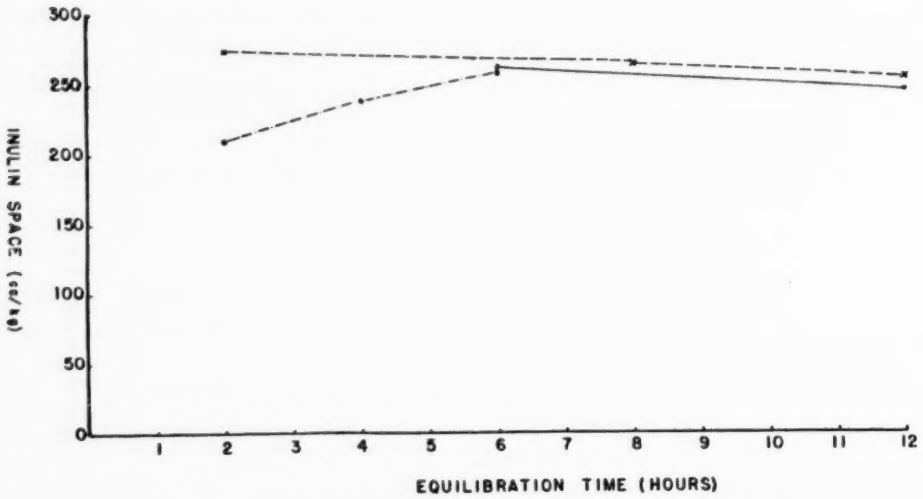

FIG. 1. REPEAT INULIN SPACES IN THREE NORMAL INFANTS WITH VARYING INFUSION PERIODS
Each type of line represents a separate subject.

infant with varying infusion times (Figure 1); 2) the time necessary for the rate of excretion of inulin to equal the constant rate of infusion was determined. Both methods revealed that equilibrium distribution in the normal infant was achieved after two to four hours of a steady infusion. A comparable infusion time has been previously demonstrated to be necessary to attain equilibrium in adults (10). In a dehydrated infant it was shown that the rate of excretion equaled the rate of infusion three hours after a continuous infusion of inulin was begun. Accordingly, the equilibrating infusion was maintained for six hours in all cases. Each infusion was prepared to provide a plasma concentration of inulin of 30 to 50 mg. per 100 ml. when delivered at a rate of 10 ± 0.15 ml. per hour with a Bowman pump. The total quantity of hypotonic salt solution administered during each space measurement was approximately 60 to 75 ml.

After the equilibrating infusion had been discontinued, the bladder was emptied and urine was collected through an indwelling catheter until all the inulin had been excreted. Complete excretion of the administered inulin in normal, dehydrated, and convalescent infants occurred within 12 to 18 hours (Figure 2). Urinary recoveries of inulin averaged 98.6 per cent (Table I).

The heavy water space was measured as the two and three hour volume of distribution of intravenously injected deuterium oxide (11). No correction was made for serum water concentration. Sufficient deuterium oxide was injected with a calibrated syringe to provide a concentration *in vivo* approximating 0.2 volumes per 100 ml. of body water. In all cases serial determinations of the serum deuterium concentration indicated that equilibrium had been attained within two hours.

Intracellular volume (non-inulin body water) was approximated as the difference between the simultaneous volumes of distribution of heavy water and of inulin. The total sodium and chloride content of the fluid phase of the extracellular compartment was estimated by multiplying the plasma sodium and chloride concentrations by the volume of distribution of inulin.

The volume of distribution of T-1824 was determined according to the method of Gregerson and Stewart using a single ten-minute blood specimen (12). Inulin and p-aminohippurate clearances were measured during the equilibrating infusion after the procedure of Smith and his co-workers (13). The bladder was washed between collection periods and at the end of the equilibrating infusion with distilled water and air.

TABLE I

*Inulin recoveries**

Patient	Age *weeks*	Inulin injected *mg.*	Inulin excreted *mg.*	Inulin recovered %
H. F.	3	240	236	98.4
M. H.	32	3410	3302	96.8
B. H.	19	1072	1041	97.2
B. H.	19	1050	1029	98.0
R. H.	4	450	475	105.0
E. L.	14	800	770	96.3

* Average per cent of inulin recovered—98.6 per cent.

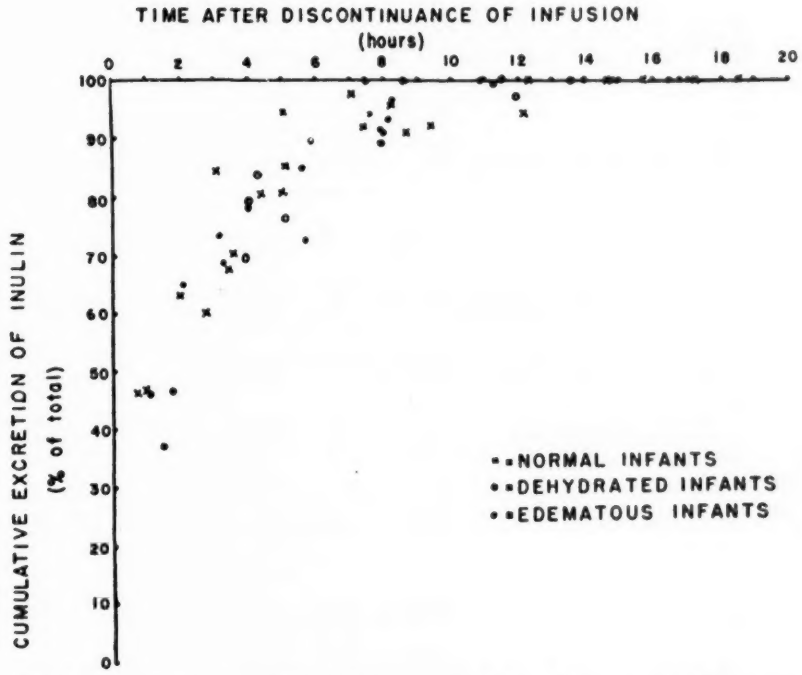

FIG. 2. COMPARISON OF THE TIME NECESSARY FOR TOTAL EXCRETION OF INULIN IN NORMAL, DEHYDRATED, AND EDEMATOUS INFANTS

TABLE II

Body water determinations in normal infants

Patient	Age *mo.*	Height *cm.*	Weight *Kg.*	Surface area m^2	Equil. time *hr.*	Volumes of distribution: T-1824 *ml.*	T-1824 *ml./Kg.*	Inulin *ml.*	Inulin *ml./Kg.*	Deuterium *ml.*	Deuterium *ml./Kg.*	Non-inulin body water *ml.*	Non-inulin body water *ml./Kg.*
O. G.	6	64.7	5.2	0.298	2			1100	210				
	6	64.7	5.2	0.298	6			1390	263				
	6	64.7	5.2	0.298	4			1260	240				
E. R.	4	52	3.1	0.205	6	203	65	870	280				
J. F.	3	52.5	3.33	0.212	6			910	273				
M. J.	3.5	48.3	5.74	0.255	6	310	54	1480	258				
S. O.	2	58.4	5.45	0.284	6	360	66	1350	248				
R. Ar.	8.5	63.5	7.48	0.344	6	400	54	1930	256	4480	597	2250	341
M. H.	7	67.3	7.71	0.360	6	361	48	2030	263	4400	570	2370	307
	7	67.3	7.61	0.358	12			1870	246				
	7	67.3	7.61	0.358						4650	619		
R. An.	4		5.23							3030	579		
B. H.	4.5	64.7	5.63	0.308	8	353	63	1500	266	4085	725	2585	459
	5	64.7	5.87	0.315	2			1610	274				
	5	64.7	5.84	0.315	12			1490	255				
T. S.	1.5	59.7	5.12	0.267	6	349	68	1380	270	3160	616	1780	346
M. F.	2.5		2.92							1870	640		
D. M.	7	58.5	6.86	0.313	6			1520	225	3755	546	2235	321
V. H.	5	66	6.66	0.335	6	375	49	1730	260	4690	703	2960	443
J. L.	2		5.03		6	270	55	1660	330	3100	616	1440	286
M. N.	3		4.31		6			1080	251				
E. J.	2	56	4.03	0.240	6	254	62	1280	310	2095	521	815	211
P. B.	2	53	4.70	0.239	6	270	53	1390	292	3410	725	2020	435
Average							58		267		610		350
Standard deviation							7		24		53		77

TABLE III

Body water distribution and renal function in infants during dehydration and subsequent recovery

Patient	Age mo.	Day of Rx	Weight Kg.	Volumes of distribution: T-1824 ml.	T-1824 ml./Kg.	Inulin ml.	Inulin ml./Kg.	Deuterium ml.	Deuterium ml./Kg.	Non-inulin body water ml.	Non-inulin body water ml./Kg.	Plasma protein conc. Gm./100 ml.	Plasma electrolyte concentration: HCO_3 mM/L.	Sodium mM/L.	Chloride mM/L.	Potassium mM/L.	Renal function: Hematocrit %	GFR ml./min.	RPF ml./min.
M. G.	3.5	0	5.84	270	46	1200	206					5.7	17.8	150	111	4.7	38		
	4	14	6.08	327	54	2750	450					6.0	25	145	102	4.5	32		
J. F.	2.5	0	3.07	180	59	550	180					6.4		155	122	4.5	30.5		
	3	12	3.20	220	69							6.4		142	103	4.5	33		
	3	17	3.33			955	287					6.4		143	99	3.4	33		
M. J.	3	0	5.70	280	49	1055	182						6.2	155	115	5	36	8	26.5
	3	2	5.90	390	66	2400	406					6.2	24	157	110	4.2	29	9.1	28
	3	8	6.10	310	50	2460	400					5.3		145	106	4.2	30	15.3	42
	3.5	16	5.74	310	54	1570	275					6.0			108		32	10.1	39.1
R. Ar.	7.5	0	7.20	380	53	1320	183	3900	543	2580	360	8.4		153	115	5.3	39.5	20.8	88
	7.5	6	7.33	450	61	2470	337	4805	656	2335	318	6.0	22.3	153	112	3.1	31	31	103
	8	14	7.33	420	58	1880	256	4520	616	2640	360	6.7		148		4.0		20.3	101
	8	25	7.48	400	54	1930	256	4480	597	2550	341			152	107	4.5	35		
R. An.	4	0	5.11	324	63	1170	229	2870	561	1700	332						32	14	46.7
	4	2	5.56	320	57	1960	352	3490	626	1530	274	5.8	26.2		105		26	24.1	
	4	7	5.23	346	66	1310	250	3030	579	1730	329								
V. H.*	5	0	6.29	287	45	1310	211					6.9	16.5	128	110	3.9	35	13.2	50.4
	5	2	6.60	375	57	1910	289	4230	640	2320	351	6.3	24.6	134	103	3.1	37	18.3	59
	5	8	6.56	380	58	2160	329	4230	645	2070	316	7.3	25.2	134	105	4.7	33	22	81
	5.5	15	6.66	325	49	1730	264	4690	703	2960	439			121	109	4.8	33		
M. N.*	3	0	3.84			660	178					7.8		134	113	5.5	43	6.5	25.7
	3	4	3.84			1000	260						16.8	125	108	5.7	35	9.6	33.2
	3	7	3.74			1030	276					6.5	26.4		107		27	7.6	32.9
	3.5	18	4.31			1080	251												
P. B.	2	0	4.08	250	64	930	228	2585	634	1655	406	6.0	11.1	130	107	3.6	31	10.5	59
	2	3	4.34	270	63	1470	339	3100	715	1530	376	5.8	21		102	4.1	31	11	32
	2	8	4.32	270	61			3325	770			5.5	21.6		102		35		
	2.5	18	4.68			1980	422	3410	727	1530	305	5.4	22.8		103		21		54
	2.5	24	4.76			1390	292											9	

* Treated with oral potassium.

The deuterium oxide content of serum water was determined by the falling drop method (14). Inulin analyses were performed by the resorcinol technique (15) and p-aminohippurate by the method of Bratton and Marshall as modified by Smith et al. (13, 16). Sodium and potassium concentrations were analyzed in an internal standard Perkin-Elmer flame photometer (Model 52A). Chloride was determined by the Van Slyke and Hiller modification of the Sendroy technique (17); total protein by the copper sulfate method (18); hematocrit with a Wintrobe tube (19); plasma carbon dioxide content by the method of Van Slyke and Neill (20).

Infants were weighed daily on a tare balance sensitive to 45 grams at a weight of 3 kilograms. Surface area was calculated from height and weight by the formula of Dubois and Dubois as graphed by Boothby and Sandiford, (21, 22).

RESULTS

A. Normal infants

The volume of distribution of deuterium oxide determined 12 times in 11 normal infants averaged 610 ml. per kilogram of body weight, with a range from 520 to 725 ml. per kilogram (Table II).

The volume of distribution of inulin, measured 19 times in 16 normal infants, averaged 267 ml. per kilogram with a range from 210 to 330 ml. per kilogram (Table II). Duplicate serial determinations of the absolute inulin space in three of these infants (O. G., M. H., B. H.) agreed within 10, 8, and 1 per cent, respectively.

In nine normal infants the non-inulin body water averaged 350 ml. per kilogram with a range from 211 to 459 ml. per kilogram (Table II).

The volume of distribution of T-1824 in 10 normal infants averaged 58 ml. per kilogram with a range from 48 to 68 ml. per kilogram (Table II).

B. Dehydrated infants

The volume of the various body water spaces in the infants during dehydration are presented in

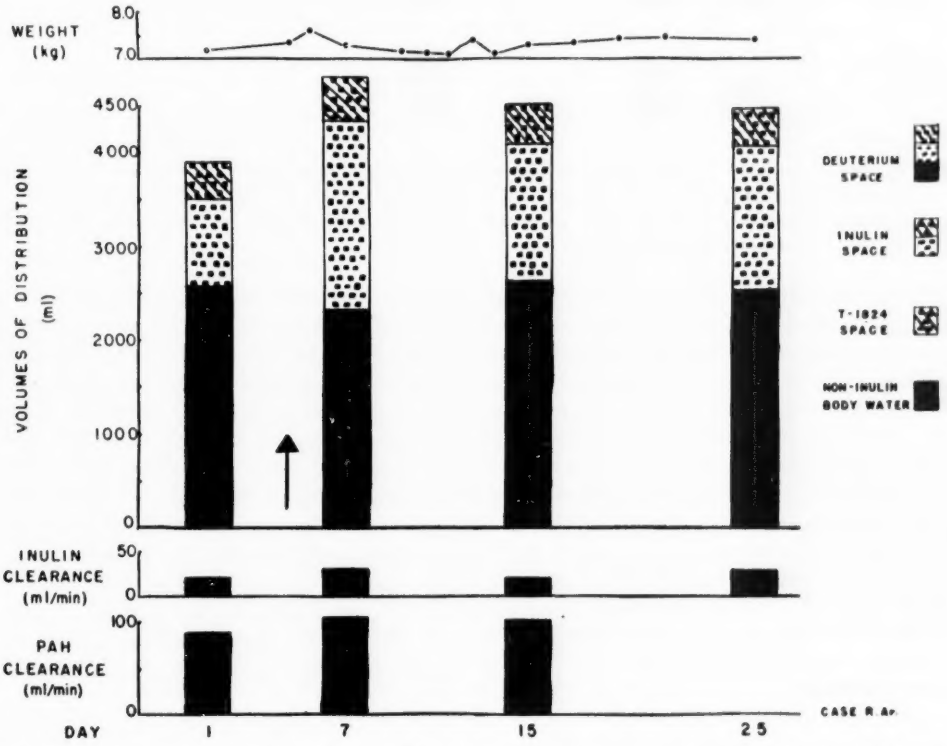

FIG. 3. SERIAL CHANGES IN BODY WEIGHT, TOTAL BODY WATER, INULIN SPACE, T-1824 SPACE, NON-INULIN BODY WATER, AND RENAL FUNCTION DURING DEHYDRATION, TREATMENT, AND RECOVERY IN SUBJECT R. AR. The arrow designates the day in which parenteral therapy was discontinued.

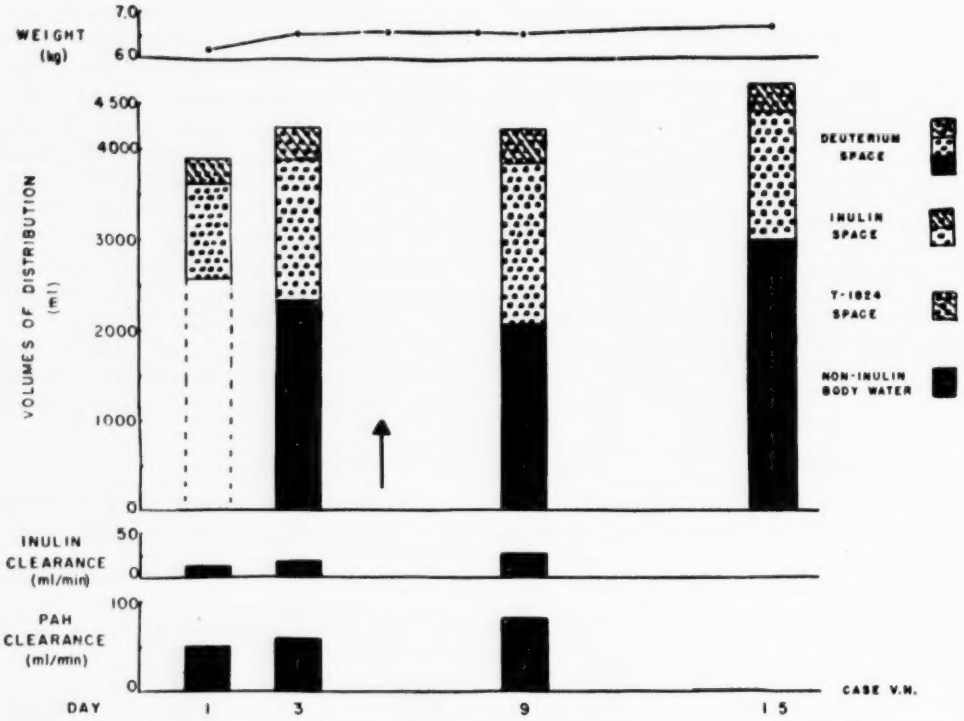

FIG. 4. SERIAL CHANGES IN BODY WEIGHT, TOTAL BODY WATER, INULIN SPACE, T-1824 SPACE, NON-INULIN BODY WATER, AND RENAL FUNCTION DURING DEHYDRATION, TREATMENT, AND RECOVERY IN SUBJECT V. H.

The arrow designates the day in whch parenteral therapy was discontinued. Dotted lines in first column indicate that total body water was not measured.

Table III. The volume of distribution of deuterium determined in three dehydrated infants averaged 580 ml. per kilogram of body weight at the time of admission, while volume of distribution of inulin in eight such subjects averaged 200 ml. per kilogram. In terms of absolute volumes, the inulin and heavy water spaces in three infants at time of admission averaged 400 and 500 ml., respectively, less than when these patients were fully recovered. The volume of the non-inulin body water in the dehydrated infants remained within the wide range of normal. The T-1824 space averaged 54 ml. per kilogram which was only 4 ml. per kilogram below the average values obtained in the same infants following recovery.

C. Infants during treatment and after recovery

Data obtained in three patients reveal that after one or two days of therapy the volume of distribution of deuterium increased to values which approximated those obtained after complete recovery (Table III). Simultaneously, the volume of distribution of inulin expanded to an average value of 367 ml. per kilogram. This represents an increment of 75 ml. per kilogram above the average value obtained when the patients were fully recovered. Three of the infants concomitantly developed puffiness of the eyelids and face. The expansion of the inulin space to a supernormal volume appeared within two days after onset of therapy. In some instances these high values persisted for 10 days after parenteral fluids were discontinued—a period of 14 days (Table III, Figures 3, 4).

The overexpansion of the volume of distribution of inulin in the early phase of therapy was not associated with a comparable increase in the volume of distribution of deuterium or of body weight.

Accordingly, during this period of treatment the non-inulin body water decreased below values present on admission (Table III, Figures 3, 4). Thereafter, during convalescence (10 days to two weeks after therapy was begun), the volume of distribution of inulin reverted to normal control values with little change in total body water or body weight (Table III). By this time the volume of the non-inulin body water thereby expanded to normal control values (Table III, Figures 3, 4). The volume of distribution of T-1824 showed only slight changes. However, the small changes observed tended to vary in the same direction as those manifested by the inulin space (Table III). From the time of admission to the time of full recovery body weight increased an average of 60 grams per kilogram (Table III). One infant (M. N.) treated with potassium was the only subject who did not display excessive expansion of the volume of distribution of inulin (Table III); however, the inulin space of the other infant so treated (V. H.) expanded to a level 430 ml. above the final recovery volume while the non-inulin body water decreased to a level 900 m. below the final volume (Table III, Figure 4).

Three infants on admission showed abnormally high plasma sodium and chloride concentrations (Table III). The other patients revealed no significant change in these concentrations. On admission the majority displayed a reduction in plasma CO_2 content. Within 48 hours these changes in electrolyte concentrations usually reverted to normal. The course of recovery of the infants with hyperelectrolytemia was indistinguishable from that of the other patients. In most instances the plasma potassium concentration diminished from a high normal range on admission to a low normal range during the first week of therapy (Table III).

On admission the electrolyte deficit of the fluid phase of the extracellular compartment averaged 60 mM of sodium and 44 mM of chloride, or 10 mM of sodium and 7 mM of chloride per kilogram of body weight below subsequent recovery values (Figure 5). The cases with hyperelectrolytemia demonstrated comparable deficits of total extra-

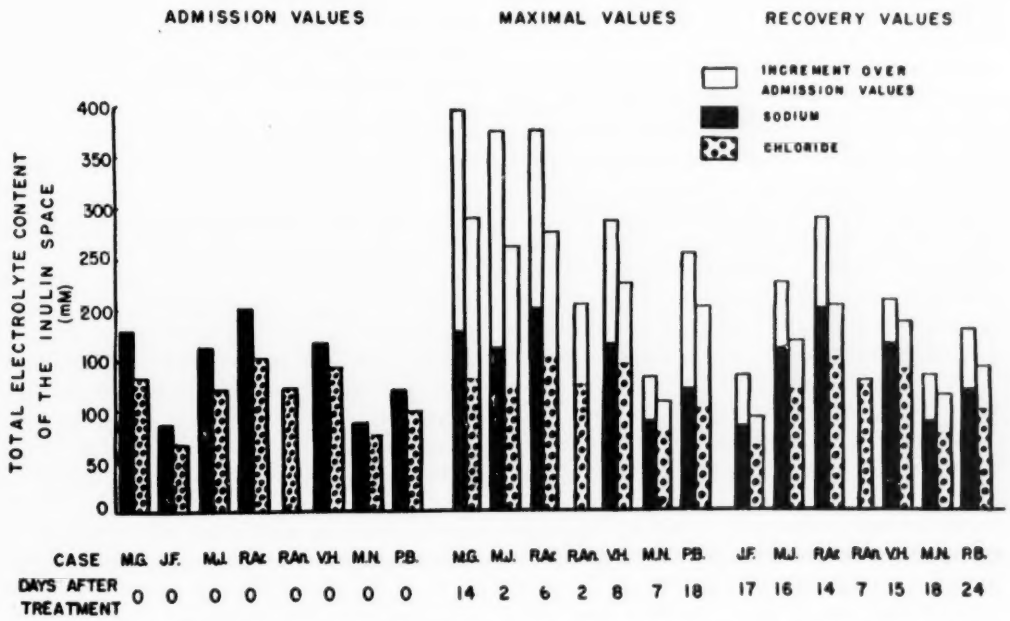
ADMISSION VALUES
MAXIMAL VALUES
RECOVERY VALUES
INCREMENT OVER ADMISSION VALUES
SODIUM
CHLORIDE
TOTAL ELECTROLYTE CONTENT OF THE INULIN SPACE (mM)
400
350
300
250
200
100
100
50
0
CASE M.G. J.F. M.J. R.Ac. R.An. V.H. M.N. P.B. M.G. M.J. R.Ac. R.An. V.H. M.N. P.B. J.F. M.J. R.Ac. R.An. V.H. M.N. P.B.
DAYS AFTER TREATMENT 0 0 0 0 0 0 0 0 14 2 6 2 8 7 18 17 16 14 7 15 18 24

FIG. 5. TOTAL ELECTROLYTE CONTENT OF THE FLUID PHASE OF THE EXTRACELLULAR COMPARTMENT (INULIN SPACE TIMES PLASMA ELECTROLYTE CONCENTRATION) DURING DEHYDRATION, MAXIMAL INCREASE DURING THERAPY AND RECOVERY

cellular sodium and chloride. At the time of maximal expansion of the volume of distribution of inulin the total sodium contained in this compartment averaged 28 mM per kilogram more than on admission and 15 mM per kilogram more than subsequent recovery values. The comparable increase of the chloride contained in this compartment averaged 17 mM per kilogram over admission values and 11 mM per kilogram over subsequent recovery values (Figure 5).

In four of six subjects prior to the onset of therapy, the inulin and p-aminohippurate clearances were slightly lower than those determined after recovery (Table III). During the early period of therapy the inulin clearance and the filtration fraction tended to reach supernormal values (considerably in excess of the final recovery values) coincident with the maximal expansion of the inulin space.

Hematocrit and plasma protein concentrations on admission were within the normal range (Table III). After the onset of therapy they usually decreased. Subsequently, the hematocrit values and plasma protein concentrations reflected the use of plasma or whole blood transfusions.

DISCUSSION

The reliability of the volumes of distribution of inulin as a measure of the fluid phase of the extracellular compartment and of the volume of distribution of deuterium oxide as a measure of the total body water has been previously discussed (23–26). These methods may be applied to the infant with little modification (see Methods). The volume of distribution of inulin and of heavy water in the control subjects (Table II) are in reasonable agreement with those reported by others (27–30).

The average expansion of the total body water detected from the time of admission to that of recovery is similar to that calculated by Darrow and his co-workers from balance studies in infants recovering from diarrhea (8). Similarly, the deficit of extracellular electrolyte exhibited on admission agrees with Darrow's data (8). However, from studies of a single infant dying from severe diarrhea, Steinitz and Weigert obtained data from which a total body water deficit of 210 ml. per kilogram may be calculated (31). This infant was undoubtedly more severely ill than those studied in our series. Indeed, it is probable that if the methods utilized in this study permitted the investigation of more severely ill infants, a greater depletion of the extracellular compartment and a consistent and significant decrease of the non-inulin fluid volume might have been detected during the stage of acute dehydration.

The overexpansion of the fluid phase of the extracellular compartment which was noted during the early period of therapy is in reasonable agreement with previous observations. Darrow and his co-workers demonstrated a significant expansion of the chloride space and a loss in the volume of "intracellular water" during the first phase of recovery from diarrhea (8). Twenty-five to fifty per cent of the infants with dehydration treated with a standard regimen displayed clinical edema comparable to that observed at the time of maximal expansion of the extracellular compartment in these subjects. Edema appearing during treatment for diarrhea has been repeatedly observed (7, 32, 33).

The sodium, chloride, and water which expanded the volume of distribution of inulin beyond values obtained after recovery may have derived from the administered fluid and electrolyte which was retained, from salt and water which was transferred from non-inulin body fluid compartments, or from both. That a load of extracellular electrolyte may be excreted only after a delay of 24 to 36 hours in the normal infant and adult has been repeatedly demonstrated (34–38). Indeed, a normal infant may retain saline given subcutaneously for as long as five days (39). Furthermore, renal function and the rate of salt excretion is reduced during the stage of hydropenia and electrolyte deficiency (40, 41). Increased adrenal cortical activity with a resultant decrease of sodium and chloride excretion may be expected under stress of infection, fever, dehydration, and trauma subsequent to admission (42). The large amounts of sodium, chloride and water retained thereby account in large part for the overexpanded volume of distribution of inulin detected during therapy.

The retention of salt and water does not, however, explain entirely the excessive increase in the volume of distribution of inulin. In the cases studied neither the volume of distribution of deuterium nor the body weight demonstrated an increment comparable to that of the inulin space. On the

average, only half of the increment of the volume of distribution of inulin was accounted for by a concomitant increase in the volume of distribution of deuterium or of body weight [5] (Table III, Figures 3, 4). In each infant in whom simultaneous inulin and deuterium spaces were measured, therefore, the non-inulin fluid volume decreased significantly early in the treatment period (Table III, Figures 3, 4). Specifically, one infant (M. J.) displayed an expansion of the volume of distribution of inulin of 1,350 ml. over the admission value after 48 hours of parenteral fluid therapy. The electrolyte content of the fluid phase of the extracellular compartment increased by 195 mM of sodium and 140 mM of chloride. However, the subject had received in this period of time only 125 mM of sodium and 90 mM of chloride, which were administered intravenously with 1760 ml. of water. Furthermore, this expansion of isotonic extracellular fluid volume persisted in some instances for 14 to 18 days, or for 10 days after discontinuance of parenteral therapy (Table III, Figures 3, 4). Late in the treatment phase, when the inulin space reverted to control values, no comparable reductions in body weight or total body water were detected. The non-inulin fluid volume therefore did not expand until the second or third week of hospitalization (Table III, Figures 3, 4). These considerations suggest that, in part at least, the prolonged over-expansion of the volume of distribution of inulin without proportionate reduction in plasma electrolyte concentrations may be attributed to an endogenous redistribution of sodium, chloride, and water into the fluid phase of the extracellular compartment.

The source of the sodium, chloride, and water transferred into the volume through which inulin is distributed is not known. It may have arisen from the cells or from any of those tissues not readily accessible to the inulin molecule. These tissues may include bone and dense connective tissue (the "solid phase of the extracellular compartment"), succus entericus, or bile (43–45). Significant quantities of salt and water may be confined in the solid phase of connective tissue (46). Large stores of labile sodium are located in the crystalline structure of bone; salt and water are contained in the gastrointestinal tract (47). The salt and water may derive, in part at least, from any one of these sources.

It is not certain why infants recovering from dehydration lose their facility for homeostatic extracellular volume control. In normal adults in whom the salt load was suddenly increased from 6 to 18 grams per day, the expansion in the volume of distribution of inulin was slight and persisted for only 24 to 48 hours (38). It has been demonstrated previously that desoxycorticosterone administration to the dog and cortisone or ACTH administration to man induces a simultaneous exogenous retention and endogenous redistribution of salt and water (11, 48). These hormones therefore may endogenously augment the extracellular volume. If hormone therapy is prolonged, redistribution is reversed and the total extracellular volume returns toward normal. These alterations are qualitatively similar to those observed in infants recovering from diarrhea. Thus it is conceivable that the concomitant redistribution and retention of salt and water observed in these infants may relate to increased adrenal cortical activity. Furthermore, recent studies have shown that an acute reduction in plasma volume may cause a rapid transfer of salt and water into the fluid phase of the extracellular compartment (49). Whether this acute redistribution is similarly mediated *via* adrenal activity is not known. In any event, the stimuli of plasma volume depletion and adrenal cortical stimulation are probably both present in infants suffering from acute diarrhea and may be related to the salt retention and redistribution that were observed in these infants.

The overexpansion of the fluid phase of the extracellular volume *per se* has not been shown to be deleterious in infants. Since continuous infusion of electrolyte solutions may be essential during the early treatment of diarrhea, the concern with possible untoward effects of an overload of electrolyte may lead to inadequate therapy. A repair solution recommended in order to avoid overtreatment (50) would have been less than adequate for repair of extracellular deficits within 48 hours in four of five cases studied. However, the decrease of the non-inulin body water during the early stage

[5] During the early phase of treatment three infants (R. Ar., R. An., P. B.) exhibited an increment in total body water which averaged 350 grams more than that in body weight. It is likely that this discrepancy reflects the utilization of body solids by sick infants maintained on inadequate caloric intakes.

of recovery may be etiologically related to those fatalities which sometimes occur during treatment after extracellular dehydration has been corrected.

One patient who received early therapy with potassium was the only subject who did not display the characteristic pattern of body fluid alterations during treatment and recovery (M. N., Table III). One other case so treated underwent a typical course, however, and in Darrow's cases those infants who displayed changes most similar to those described here were subjects who received early potassium therapy (8). Whether the administration of potassium will regularly inhibit the progressive depletion of non-inulin body fluid is not yet known. Insofar as these stores of sodium and chloride derive from the solid phase of the extracellular compartment—bone and connective tissue (51)—one would not expect the administration of potassium to inhibit such a redistribution. Although a reduction in plasma volume was present during the stage of dehydration (Table III) neither this volume nor the hematocrit nor the plasma protein concentration afforded a reliable index of the diminution in the volume of the fluid phase of the extracellular space (11).

The association of increased glomerular filtration rate with an expanded volume of distribution of inulin has been previously observed under hormonal influence (11, 48). The retention of salt and water despite the concomitant increase in glomerular filtration rate reflects the enhanced rate of tubular resorption of salt during early therapy. This finding similarly suggests that increased adrenocortical activity may underlie the alterations which were noted.

SUMMARY AND CONCLUSIONS

1. In the normal infant two to eight months of age, the T-1824, inulin, and deuterium volumes of distribution averaged 58, 267, and 610 ml. per kilogram, respectively.

2. In infants suffering from acute, moderately severe dehydration, there was a considerable diminution of total body water due chiefly to extracellular fluid loss. Early in the treatment phase (two to 14 days after the onset of therapy) the inulin space expanded to supernormal volumes. This overexpansion of the inulin space was not associated with a comparable increase in the deuterium space or of body weight. During convalescence (two to three weeks after the onset of therapy), a reduction of the inulin space to normal values without comparable fall in body weight or deuterium space was observed.

3. The T-1824 space increased to normal within two to three days after the onset of therapy.

4. Glomerular filtration rate tended to be low during the state of dehydration. Thereafter, the inulin clearance increased to levels higher than those observed after recovery. The maximal increase in filtration rate and inulin space coincided.

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THE CARDIAC OUTPUT AT REST IN LAENNEC'S CIRRHOSIS [1]

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INTRODUCTION

Patients with Laennec's cirrhosis and those with occidental beri-beri frequently have the common background of consumption of alcoholic beverages and inadequate diet of long duration. A short circulation time which may be found in occidental beri-beri (1) and in Laennec's cirrhosis (2, 3) suggests an elevated cardiac output. In the case of beri-beri, this has been verified (4, 5, 6, 7), but, to our knowledge, direct measurements of systemic blood flow have not been reported in chronic liver disease. Increased flow of blood to the periphery in patients with Laennec's cirrhosis is suggested by the frequent occurrence of warm extremities, cutaneous vascular spiders, wide pulse pressure, and capillary pulsations in the nail beds.

The present study reports measurements of the resting cardiac output, blood pressure, and peripheral resistance in patients with chronic alcoholism and disease of the liver.

MATERIALS AND METHODS

Selection of patients

Twenty-two hospitalized patients [3] with clinical and/or laboratory evidence of chronic parenchymal disease of the liver were studied. Only patients without clinical evidence of co-existing heart disease and without evidence of acute gastro-intestinal bleeding were included. All subjects gave a history of excessive intake of alcoholic beverages and an irregular and inadequate diet of long-standing.

Of the 22 patients studied, three had "fatty liver," 11 had Laennec's cirrhosis without fluid retention, and eight had Laennec's cirrhosis with either ascites or peripheral edema or both. Of the 19 patients with Laennec's cirrhosis, all save one (No. 17) had enlargement of the liver, and all but two (Nos. 11 and 18) had cutaneous vascular spiders. All 22 patients had abnormally high Bromsulfalein retention.

A diagnosis of "fatty liver" was made clinically when there was enlargement of the liver and laboratory evidence of hepatic dysfunction without the following: icterus, cutaneous vascular spiders, ascites, edema, splenomegaly, or prominent abdominal veins.

PROCEDURES

Each patient was studied early in the morning without any premedication, fasting and recumbent. An indwelling needle was inserted into the left brachial artery and a similar needle was placed into a right antecubital vein. The patient then was allowed to rest for at least twenty minutes.

Cardiac output

The cardiac output was determined by the dye-injection method as described by Hamilton and his co-workers (8). Five mg. of a 0.5 per cent solution of Evans blue dye were injected rapidly through the venous needle from a tuberculin syringe previously calibrated by weighing. The dead space of the indwelling needle was known and accounted for in determining the amount of dye injected. Samples of arterial blood were collected from the arterial needle through a short length of polyethylene tubing into heparinized tubes mounted along the rim of a circular lucite disc equipped with funnels arranged so that spillage between tubes was minimized. The disc was rotated about a centrally placed vertical shaft by a variable-speed electric motor. The usual collection time for each tube was two and one-half seconds. Samples of arterial blood were centrifuged for 20 minutes at 3,000 rpm. and the concentration of dye in undiluted plasma was determined in micro-cells of 0.5 cc. capacity at a wave length of 625 mμ. using a Beckmann Model DU spectrophotometer. In most patients duplicate estimations were made.

The cardiac index was calculated from the cardiac output and the body surface area using the height and weight at time of study irrespective of the presence or absence of edema. In subjects with marked obesity and perhaps in those with abnormal fluid retention, the body surface area calculated according to Du Bois and Du Bois (9) might distort the predicted value for cardiac index (10, 11). The cardiac output also was expressed in terms of oxygen consumption as a ratio by the formula:

$$\frac{\text{Cardiac output L./min.} \times 100}{\text{Oxygen consumption cc./min.}}$$

[1] Presented in part at the Eastern Section Meeting of the American Federation for Clinical Research, December 5, 1952, New York, N. Y.

[2] Postdoctoral Research Fellow, Life Insurance Medical Research Fund.

[3] We appreciate the courtesy of Dr. Charles S. Davidson and his associates, and of the various medical services of the Boston City Hospital for making their patients available for study.

TABLE I

*Cardiac index—"normal" values**

Authors	No. subjects	Method	Mean ± Standard deviation of sample	
			Cardiac index	Cardiac output L./min. × 100 / Oxygen consumption cc./min.
Cournand et al. (J. Clin. Invest., 1945, **24**, 106.)	30	Fick	3.31 ± 0.54	2.32 ± 0.32
Stead et al. (J. Clin. Invest., 1945, **24**, 326.)	18	Fick	3.27 ± 0.57	2.52 ± 0.39
Ebert et al. (J. Clin. Invest., 1949, **28**, 1134.)	12	Fick	3.59 ± 0.72	2.64 ± 0.34
Chapman et al. (J. Clin. Invest., 1950, **29**, 651.)	7	Fick	3.27 ± 0.62	2.67 ± 0.43
Dexter et al. J. Applied Physiol., 1951, **3**, 439.)	7	Fick	3.79 ± 0.83	2.77 ± 0.37
Doyle et al. (J. Clin. Invest., 1951, **30**, 345.)	10	Fick Dye	3.52 ± 0.79 2.85 ± 0.40	2.41 ± 0.35 1.97 ± 0.87
Freis et al. (J. Clin. Invest., 1952, **31**, 131.)	8	Dye	4.19 ± 0.81	
Present Authors	12	Dye	3.76 ± 0.65	

* Calculated by us from data in sources listed. Subjects with "anxiety" excluded as in individual reports. Two or more observations on the same subject were averaged, and the mean was used in these calculations.

Table I indicates values of cardiac index as reported by others and as determined in this laboratory using the dye-injection method in subjects who were considered essentially normal. Also shown are values for the ratio of cardiac output to oxygen consumption.

Oxygen consumption

Oxygen consumption was determined over a six-minute period by means of a closed circuit water spirometer filled with oxygen, after the cardiac output studies were completed.

Arterial pressure

Phasic and mean arterial pressures were measured directly by means of an electromanometer of the condenser-microphone type and recorded on a direct writing oscillograph.[4]

Peripheral resistance

The formula used was (12):

$$\text{Resistance} = \frac{\text{Mean arterial pressure mm. Hg} \times 1332}{\text{Cardiac output cc./sec.}}$$

[4] Made by Sanborn Company, Cambridge, Massachusetts.

Venous pressure

Venous pressure was estimated by means of a saline manometer attached to the indwelling needle located in the antecubital vein, and referred to a zero point 5 cm. below the angle of Louis.

RESULTS

The data are found in Table II.

Cardiac output (Figure 1)

The mean cardiac index of patients with disease of the liver was 4.26 L. per min. per M^2, and the standard deviation of the sample was ± 2.73 L. per min. per M^2. Values below the normal range were not seen, and in seven patients they were above the normal range.

As indicated in Figure 1, elevations of output were noted in one of the three patients with "fatty liver" as well as in patients with cirrhosis, irrespective of the presence or absence of abnormal fluid retention or of jaundice.

TABLE II

Cardiovascular studies in patients with cirrhosis

Subject* No.	Age	Sex	B.S.A. M^2	Days in hospital at time of study	Icterus† index units	B.S.P. ret. 5 mg. Kilo. 45 min. %	Hct. %	Oxygen cons. S.T.P. *cc./min.*	Heart rate *per min.*	Cardiac index *L./min./M²*	Mean	Cardiac‡ output ÷ oxygen cons.	Mean stroke volume *cc.*	Blood press. Syst./Diast. *mm. Hg*	Mean	Peripheral resistance *dynes cm. sec.⁻⁵*	Transverse§ cardiac diameter % predicted	K‖	Venous pressure *mm. saline*
"Fatty liver"																			
1	45	M	1.78	1	<10		43	289	84	5.07 6.68	5.88	3.62	126	114/68	88	672	90	.438	
2	44	M	1.77	2	10–20	33	43	256	86	6.10 3.54	4.82	3.33	100	140/90	107	1002		.406	30
3	41	M	1.70	1	<10	12	39	269	95	3.46 3.68	3.57	2.27	64	153/85	112	1466	99	.370	40
Laennec's cirrhosis without edema or ascites																			
4	40	F	1.67	77	<10	30	30	191	100	6.05 6.11	6.08	5.33	101	113/60	83	654	108	.463	90
5	42	M	1.79	22	20–30	54	38	292	87	5.70 6.25	5.98	3.66	123	105/60	80	598	112	.425	65
6	49	F	1.92	23	<10	27	29	251	79	4.86 5.47	5.17	3.95	125	93/57	74	596	98	.471	100
7	34	F	1.48	19	>100	45	32	243	80	5.07 4.78	4.93	3.00	91	108/61	80	878	109	.394	90
8	44	M	1.76	24	30–40		38	322	76	4.62	4.62	2.52	107	115/70	85	836	119	.394	
9	40	M	1.87	102	<10	17	43	288	88	4.12	4.12	2.67	87	100/74	83	862	119	.438	
10	43	M	1.64	28	10–20	48	40	295	100	3.91	3.91	2.17	64	102/82	89	1110	123	.412	10
11	51	M	1.63	14	<10	17	34		62	3.33 3.27	3.30		87	130/80	97	1439	102	.400	
12	46	M	1.40	22	10–20	39	44		90	3.04	3.04		47	118/70	86	1617	104		
13	57	M	1.85	1	<10		48	246	73	3.08 2.95	3.02	2.27	76	120/75	90	1289	113	.530	
14	42	M	1.77	1	10–20	55	41	304	98	2.50 3.01	2.76	1.61	50	130/100	120	1965		.411	

TABLE II—*Continued*

Subject* No.	Age	Sex	B.S.A. M^2	Days in hospital at time of study	Icterus† index units	B.S.P. ret. 5 mg. Kilo. 45 min. %	Hct. %	Oxygen cons. S.T.P. *cc./min.*	Heart rate *per min.*	Cardiac index *L./min./M²*		Cardiac‡ output ÷ oxygen cons.	Mean stroke volume *cc.*	Blood press. Syst./Diast. *mm. Hg*	Blood press. Mean	Peripheral resistance *dynes cm. sec.⁻⁵*	Transverse§ cardiac diameter % predicted	K‖	Venous pressure *mm. saline*
											Mean								
Laennec's cirrhosis with edema and/or ascites																			
15	42	M	2.15	27	<10	47	32	257	105	11.2	11.2	9.40	233	125/105	111	367	114	.430	
16	59	M	1.77	2	20–30	45	40	332	94	6.33 5.76	6.05	3.23	114	111/69	88	658	111	.446	80
17	51	F	1.94	1	40–60	45	30		117	5.32 4.84	5.08		84	100/67	76	618	102	.431	30
18	64	M	2.06	37	10–20	39	33	253	74	5.39 4.31	4.85	3.98	136	100/55	68	542		.422	90
19	38	F	1.65	4	<10		27	200	97	4.37 4.11	4.24	3.50	72	78/56	66	754	102	.428	50
20	39	M	1.85	3	30–40	38	21	238	120	4.61 3.46	4.04	3.13	62	104/66	76	814	135	.459	110
21	42	M	1.88	25	20–30	34	38	270	76	3.97	3.97	2.76	98	97/72	80	854	113	.390	
22	62	M	2.04	15	<10	61	31		93	3.04 3.34 3.34	3.24		71	160/95	117	1408		.479	

* Vitamin B_1 therapy: Subject 4, 20 mg. I.M., 30 days; Subject 5, 10 mg. I.M., 22 days; Subject 19, 100 mg. I.M., 4 days; Subject 21, 100 mg. p.o., 21 days. Subjects 4, 5, 12, and 21 were given a daily multivitamin supplement which contained thiamine hydrochloride 10 mg., riboflavin 6 mg., niacinamide 80 mg., pyrodoxine HCl 6 mg., calcium pantothenate 6 mg., and ascorbic acid 100 mg.

† Icterus index, less than 10 considered normal.

‡ $\frac{\text{Cardiac output L./min.} \times 100}{\text{Oxygen consumption cc./min.}}$

§ Criteria of Ungerleider, H. E., and Gubner, R., Am. Heart J., 1942, **24**, 494.

‖ Constant for Q-T interval corrected by the formula of Bazett.

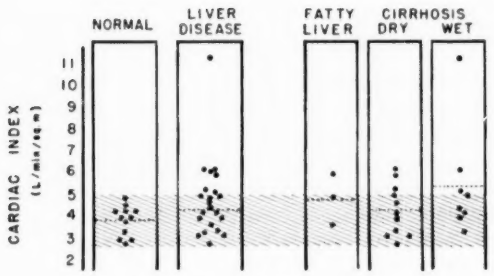

FIG. 1. SCATTER OF CARDIAC INDEX

The second column represents all patients with liver disease. The third, fourth, and fifth are subdivisions of column two. The mean for each group is indicated by the dotted line. The shaded area represents the normal mean ± two standard deviations of the sample.

Although one patient (No. 15) had a very high cardiac index, 11.2 L. per min. per M^2, this apparently did not unduly influence the mean of 4.76 since the median value for the series lay between 4.62 and 4.24 L. per min. per M^2. (This individual was restudied six weeks later at which time his cardiac index was 16.3 L. per min. per M^2 and his oxygen consumption was 263 cc. per min.)

The mean value of the ratio,

$$\frac{\text{cardiac output L./min.} \times 100}{\text{oxygen consumption cc./min.}},$$

in the patients with liver disease was 3.47 and ranged from 1.61 to 9.4. In contrast the mean values of this ratio in normal subjects (Table I) range from 1.97 to 2.77. Elevated ratios were found in patients with cirrhosis with and without fluid retention and in patients with "fatty liver."

Figure 2 is a plot of cardiac index against the cardiac output-oxygen consumption ratio. Those patients with the highest value of cardiac index had the highest ratios, suggesting that where the cardiac output is elevated such elevations are not accompanied by parallel increases in oxygen consumption.

Arterial pressure

Values for mean arterial pressure ranged from 66 to 120 mm. Hg. Patients with the higher pressures generally had normal outputs with one exception (No. 15), whose mean pressure was 111 mm. Hg at a time when his cardiac index was 11.2 L. per min. per M^2.

Peripheral resistance

Values ranged from 367 to 1965 dynes-cm.-seconds^{-5} with a mean of 955, a value within accepted normal standards (13). Peripheral resistance was inversely related to cardiac output as seen in Figure 3. The mean resistance of the seven patients with high cardiac outputs was 596, a low value.

Venous pressure

Antecubital venous pressures where measured were within normal limits. Neck veins were not distended in recumbency in any patients.

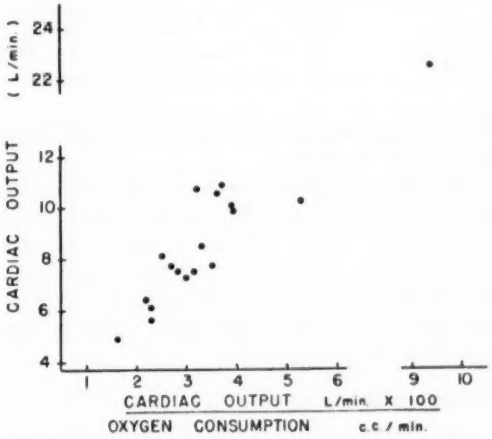

FIG. 2. RELATION OF CARDIAC OUTPUT TO OXYGEN CONSUMPTION AT DIFFERENT LEVELS OF CARDIAC OUTPUT

Heart rate and stroke volume (Figure 4)

A slight elevation of resting heart rate was a feature of most patients with liver disease. Although the patients with the highest outputs tended to have faster rates, there was no consistent association between output and rate. Elevations of stroke volume as opposed to tachycardia were the immediate mechanism which produced the elevations in output.

Heart size

With the exception of two individuals (Nos. 17 and 22), all patients were examined by fluoroscopy. No chamber enlargement was noted. Unusually vigorous cardiac pulsations were conspicuous in

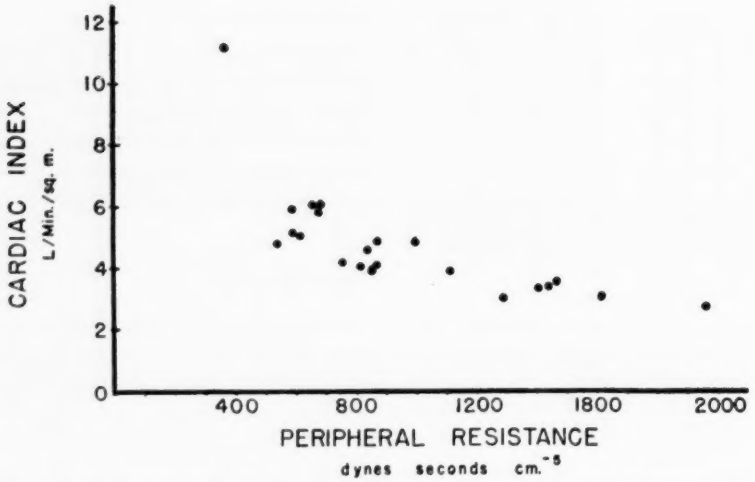

FIG. 3. RELATION OF TOTAL PERIPHERAL VASCULAR RESISTANCE TO CARDIAC INDEX

some patients and frequently were associated with large stroke volumes.

The transverse diameter of the heart measured from teleoroentgenograms averaged 110 per cent of predicted values. (Normal range = 90 per cent to 110 per cent.) The mean transverse diameter in the seven patients with elevated outputs averaged 105 per cent of predicted values. Heart size, then, was not abnormal.

Electrocardiograms

The Q-T interval was prolonged in eight patients using the criteria of Shipley and Hallaran (14). Four of the eight had a high cardiac output. All electrocardiograms otherwise were within normal limits.

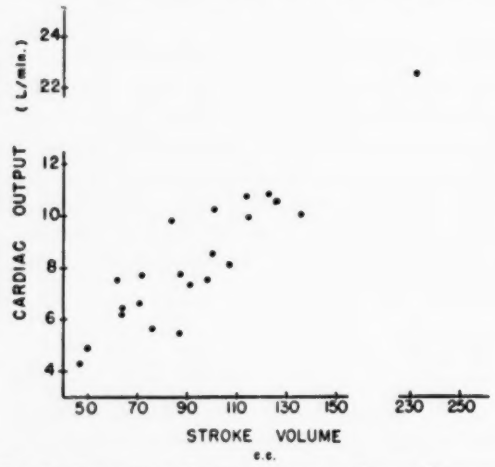

FIG. 4. RELATION OF STROKE VOLUME TO CARDIAC OUTPUT

DISCUSSION

The data indicate that the cardiac output determined at rest and in recumbency may be normal or distinctly elevated in patients with chronic alcoholism and parenchymal disease of the liver.

When the output is increased, it is out of proportion to the oxygen consumption or, by implication from the Fick formula (C.O. = oxygen consumption/arterio-venous oxygen difference), the arterio-venous oxygen difference must be decreased. The increase in blood flow is mainly accomplished by a larger output per beat. Since the arterial pressures are essentially normal, the increased blood flow is associated with decreased total vascular resistance. The hemodynamic picture in these subjects with elevated outputs resembles the high output state as seen in beri-beri heart disease (4, 5, 6, 7) and in the "shunt circulation" of arterio-venous fistulae (15, 16) or Paget's disease (17).

Several factors might be involved in producing the elevated outputs seen in one-third of the present series:

Anxiety, which may elevate the cardiac output (13, 18, 19), does not appear responsible for the increase in blood flow since the subjects were relaxed, indifferent, and without overt resentment.

Anemia, a frequent feature of parenchymal disease of the liver, is associated with an elevation of cardiac output when the hemoglobin falls to about 7 Gm. per 100 cc. or the hematocrit to about 20 per cent (20). Anemia of such degree was not seen in these patients, and since high values for cardiac output were seen in subjects with normal or near normal hematocrits, anemia does not adequately explain the elevated output.

All subjects gave a history of longstanding consumption of alcohol combined with a poor dietary intake. This background is similar to that of patients with occidental beri-beri as reported by Weiss and Wilkins (1) and by Blankenhorn (21). However, in our patients, clinical evidence of thiamine deficiency, such as neuritis, was absent, venous pressure was not elevated, cardiac enlargement was not evident, and heart failure was not seen. Four of the seven subjects with elevated outputs (Nos. 4, 5, 6, and 15) had been hospitalized 22 days or longer before study, and during this time they consumed nutritious diets under supervision. Two of these four patients (Nos. 42 and 5), in addition, were given supplementary thiamine chloride parenterally (Table II). In view of these facts, occidental beri-beri seems unlikely as an explanation of the elevated outputs seen in some patients.

Elevated concentrations of blood pyruvic and lactic acid have been noted in some patients with cirrhosis (22, 23, 24). This raises the possibility of impaired utilization of thiamine even in the presence of adequate amounts of the vitamin.

The high output state noted in some patients may be related to a deficiency state other than beri-beri. However, Howarth (25) studied the resting cardiac output in 20 severely malnourished individuals with hunger edema, and without evidence of vitamin deficiencies. The outputs were normal or low. The nutritional heart disease described in association with liver disease in the South African Bantu (26) also appears associated with normal or low cardiac outputs at least at the stage of the disease observed.

The existence of arterio-venous communications in normal as well as in cirrhotic livers has been demonstrated by Herrick, by Prinzmetal and his co-workers, and by Dock (27, 28, 29). The role of these communications in producing elevations of cardiac output would not seem great since estimates of hepatic blood flow and splanchnic arterio-venous oxygen difference (30, 31) suggest the former is decreased and the latter increased in subjects with cirrhosis.

The site of the increased blood flow thus would seem to lie in the periphery of the body, and we fall back on the concept suggested by Weiss and Wilkins (1) in explanation of the circulatory changes in beri-beri, namely that there exists generalized peripheral arteriolar dilatation, acting in effect like multiple arterio-venous shunts in parallel.

Shorr, Zweifach, Furchtgott, and Baez (32) have demonstrated that a vasodilator material, VDM, is produced by the liver under anaerobic conditions, and that it is inactivated by normal but not by anoxic liver tissue. Moreover, VDM was not inactivated under aerobic conditions by the livers of cirrhotic rats (33). It is thus conceivable that in human cirrhosis VDM is increased, or there is an imbalance in the VDM-VEM system in favor of VDM. This, then, could be a mechanism involved in the production of peripheral vaso-dilatation.

In the final analysis, we do not know whether the elevated outputs are related specifically to hepatic disease, or to a form of chronic malnutrition, or even whether they may represent the circulatory response of certain patients with chronic alcoholism to the laboratory situation.

The high incidence of prolongation of the Q-T interval is of interest in relation to similar electrocardiographic findings observed in occidental beri-beri (1), and in general deficiency states not related to thiamine deficiency (34). Prolongation of the Q-T interval has been noted by other workers in cirrhosis (35, 36).

SUMMARY

Observations made in 22 patients with a history of alcoholism, and inadequate diet, and cirrhosis of the liver indicate that the resting cardiac output was elevated in one-third of the patients and was associated with a large stroke volume, normal blood pressure, and low peripheral vascular resistance. Where the cardiac output was high, it was elevated out of proportion to the oxygen consumption, indicating a decreased arterio-venous oxygen difference.

It is suggested that dilatation of the peripheral vascular bed is responsible for the increased blood flow.

ACKNOWLEDGMENTS

We are grateful for the technical assistance of Miss Bernice E. Fingerman.

We express our gratitude to Dr. Laurence B. Ellis for his encouragement and advice throughout this investigation.

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A NEW METHOD FOR ESTIMATING SERUM ACID PHOSPHATASE OF PROSTATIC ORIGIN APPLIED TO THE CLINICAL INVESTIGATION OF CANCER OF THE PROSTATE [1, 2]

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Since the studies of Gutman and Gutman (2, 3), measurements of the serum acid phosphatase have become a recognized and widely used ancillary procedure for the diagnosis of cancer of the prostate and a practical means for the evaluation of the functional status of the tumor in many patients with metastasizing cancer of the prostate. While an abnormally increased value of serum acid phosphatase is considered to be a specific index for the presence of carcinoma of the prostate, a normal serum acid phosphatase value in itself cannot be interpreted as signifying absence of this disease. According to Bodansky and Bodansky (4) in their review of the literature, only 24 per cent of unselected patients without and 81 per cent with bone metastases from cancer of the prostate show elevated serum acid phosphatase values. Thus, 20 to 75 per cent of proven cases of cancer of the prostate show equivocal or normal serum acid phosphatase levels.

Attempts have been made to render the determination of serum acid phosphatase more specific for cancer of the prostate. Thus, a search has been made for substrates which would be split preferentially by the prostatic acid phosphatase in the serum (5). In addition, limited success in the improvement of clinical performance has been claimed by Herbert (6) for the use of ethyl alcohol as an inhibitor of prostatic acid phosphatase and by Abdul-Fadl and King (7) for formalin inhibition of interfering red cell acid phosphatase present in hemolyzed sera.

Fishman and Lerner recently reported a method for estimating serum acid phosphatase of prostatic origin (8) which is based on the inhibitory action of L-tartrate on prostatic acid phosphatase (9). It was found that activity of prostatic acid phosphatase added *in vitro* to serum could be quantitatively measured and that under the circumstances existing in humans, only a small fraction of the serum acid phosphatase arose from the prostate. This fraction was increased in the presence of cancer of the prostate (8).

This paper constitutes a report of the clinical experience obtained with this new method for the determination of serum acid phosphatase of prostatic origin in some 200 patients, including twenty-one subjects with proven prostatic carcinoma.

METHODS AND CLINICAL MATERIAL

The present method for estimating prostatic acid phosphatase in serum yields two values; one is the so-called "Total" (T) acid phosphatase expressed in King-Armstrong units (mg. phenol liberated in one hour from phenylphosphate under standardized conditions), and the other is "Prostatic" (P) acid phosphatase expressed in the same K. A. units, but representing that amount of the total serum acid phosphatase which is inhibited by L-tartrate. All sera were assayed for both T and P serum acid phosphatase.

The aged control subjects were hospitalized at the Holy Ghost Hospital, Cambridge, and at the Jewish Memorial Hospital, Roxbury. Ambulatory patients were studied at the Boston Dispensary. Long term study of pa-

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[2] Presented before the Forty-Fourth Annual Meeting of the American Association for Cancer Research (1), April 9, 1953.

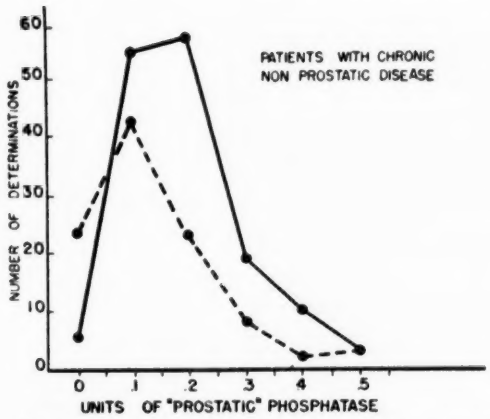

FIG. 1. A STUDY OF THE DISTRIBUTION OF VALUES FOR "PROSTATIC" ACID PHOSPHATASE IN A POPULATION OF ELDERLY, CHRONICALLY-ILL PATIENTS

The solid line represents data obtained in males and the broken line, in females.

tients with cancer of the prostate was carried out mainly at the Holy Ghost Hospital, Cambridge. Most of the surgical cases were studied at the New England Center Hospital.

The normal or non-pathological range for total acid phosphatase is here taken as 0.5 to 5.0 K. A. units.

In view of our yet limited experience with the new method, *tentative* working standards have been used in order to evaluate the clinical worth of the method. Accordingly at this time normal limits for prostatic acid phosphatase are given as 0 to 0.5 units and abnormal values begin with 0.6 units.

Data on the reproducibility of the analytical method have been published (8).

In the cases with "proven cancer of the prostate" this diagnosis was based on the histopathological examination of prostatic tissue unless otherwise stated. In some cases where no biopsy could be performed the findings of a stony hard nodular prostate with bone metastases and elevated total acid phosphatase were accepted as sufficient evidence that cancer of the prostate was present (Patients B., C., D., and H.).

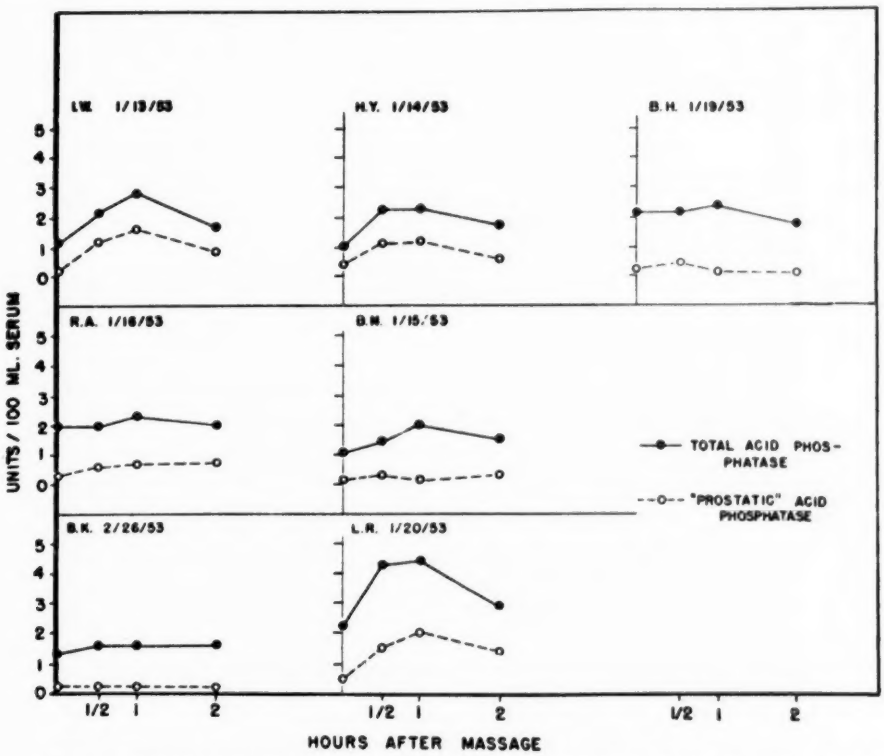

FIG. 2. EFFECT OF PROSTATIC MASSAGE ON SERUM TOTAL AND "PROSTATIC" ACID PHOSPHATASES

Patients B. H. and I. W. were massaged for five minutes, the remaining subjects for two minutes. B. H. at an earlier date had undergone a prostatectomy.

TABLE I

Serum acid phosphatase in conditions other than cancer of the prostate*

Prostatic disease									Cancer				Non-prostatic disease							
Benign prostatic hypertrophy						Chronic prostatitis							Non-cancer							
Patient No.	Total	Prostatic	Patient No.	Total	Prostatic	Patient No.	Total	Prostatic	Patient No.	Disease	Total	Prostatic	Patient No.	Disease	Total	Prostatic	Patient No.	Disease	Total	Prostatic
1	1.6	0.3	23	1.4	0.2	45	1.4	0.4	67	Cancer of bladder	1.5	0.5	76	Neurological disease	0.7	0.1	100	Chronic pancreatitis	1.2	0.2
2	1.1	0.2	24	1.4	0.1	46	0.6	0.1	68	Cancer of bladder	1.3	0.1	77	Neurological disease	0.6	0.1	101	Gaucher's disease	1.9	0.0
3	1.6	0.3	25	1.5	0.2	47	0.4	0.1	69	Cancer of pancreas	0.7	0.1	78	Neurological disease	0.5	0.2	102	Gaucher's disease	2.3	0.2
4	0.7	0.3	26	1.4	0.1	48	1.2	0.4	70	Cancer of pancreas	0.9	0.1	79	Neurological disease	0.7	0.1	103	?	1.3	0.1
5	1.9	0.2	27	0.7	0.2	49	0.9	0.4	71	Cancer of antrum	0.5	0.4	80	Neurological disease	0.7	0.4	104	?	0.7	0.5
6	0.8	0.1	28	1.1	0.2	50	1.0	0.1	72	Adenocarcinoma of intestine	0.7	0.2	81	Neurological disease	1.0	0.1	105	?	1.4	0.1
7	0.2	0.0	29	0.9	0.0	51	1.1	0.6					82	Hyperparathyroidism	2.3	0.5	106	?	1.8	0.2
8	0.2	0.0	30	1.5	0.3	52	1.7	0.2	73	Cancer of liver	0.9	0.3					107	Lithiasis of kidney	0.6	0.1
9	0.8	0.1	31	1.4	0.3	53	1.1	0.2	74	Cancer of lung (bone metast.)	3.2	0.3	83	Hyperparathyroidism	1.4	0.2	108	Glomerulonephritis	1.9	0.2
10	1.5	0.1	32	1.1	0.5	54	1.0	0.3									109	Stricture of urethra	2.0	0.0
11	1.1	0.3	33	1.0	0.3	55	1.7	0.1	75	Hypernephroma (bone metast.)	1.2	0.3	84	Cholecystitis	1.5	0.1	110	Stricture of urethra	0.9	0.0
12	0.6	0.4	34	2.0	0.0	56	1.6	0.2					85	Paraplegia	1.2	0.3	111	Stricture of urethra	2.2	0.0
13	1.3	0.5	35	1.7	0.3	57	1.1	0.1					86	Cor pulmonale	0.9	0.0	112	Stricture of urethra	2.4	0.2
14	0.8	0.1	36	2.1	0.2	58	1.4	0.1					87	Uremia	1.8	0.4	113	Stricture of urethra	1.2	0.2
15	0.9	0.1	37	1.2	0.1	59	1.3	0.5					88	Cirrhosis	1.2	0.1	114	Stricture of urethra	1.1	0.0
16	1.1	0.2	38	1.2	0.1	60	1.4	0.0					89	Cirrhosis	2.0	0.7	115	Urethral stricture	1.3	0.3
17	1.3	0.1	39	1.1	0.3	61	1.7	0.5					90	Ulcerative colitis	1.4	0.5			1.6	0.2
18	0.2	0.1	40	2.8	0.4	62	1.0	0.2					91	Fatty liver	0.6	0.0	116	Tuberculosis	1.1	0.3
19	0.7	0.3	41	1.8	0.4	63	1.3	0.1					92	Bowel obstruction	1.1	0.7	117	Tuberculosis	1.3	0.2
20	0.8	0.4	42	0.7	0.1	64	1.7	0.4					93	?	0.7	0.3	118	Lues (CN)	1.4	0.1
21	0.8	0.1	43	1.0	0.3	65	2.1	0.1					94	?	1.3	0.3	119	Osteoporosis	1.2	0.3
22	2.5	0.4	44	1.7	0.1	66	2.1	0.4					95	?	1.3	0.2	120	Cerebral vascular accident	0.9	0.3
													96	Alcoholism	1.5	0.4	121	Septicemia	1.1	0.3
													97	Gastrointestinal hemorrhage	0.4	0.1	122	Genitourinary disturbance	0.9	0.3
																	123	Carbuncle of urethra	1.4	0.3
													98	Gastrointestinal hemorrhage	0.7	0.2				
													99	Chronic pancreatitis	1.2	0.3				

* Total and prostatic acid phosphatase are expressed in King-Armstrong units per 100 ml. serum.

TABLE II

Serum acid phosphatase in proven prostatic carcinoma*

Untreated patients					Treated patients					
Patient No.	Comments	Days†	Total	Prostatic	Patient No.	Operation	Comments	Days†	Total	Prostatic
					Surgery					
124	Metastases		29.5	26.1						
		14	52.5	50.5	130		No metastases		1.3	0.7
125	Metastases		23.0	19.5				4	2.3	1.3
126	Metastases		20.6	17.0		(27 days post orchiectomy)		31	1.4	0.6
127	Metastases		34.5	30.3				120	0.8	0.2
128	Metastases		43.0	32.6	131		? Metastases		2.3	0.7
		9	26.4	24.8		(3 days after radical prostatectomy)		4	1.5	0.6
		19	34.1	30.5				5	1.7	0.5
129	Metastases		19.1	16.5	136		Pre-operative 7/21		9.6	5.9
130	No metastases		1.3	0.7			Pre-operative 7/31		11.7	8.8
131	? Metastases		2.3	0.7		Post adrenalectomy	8/26		6.5	2.4
132	No metastases		1.7	1.0			9/19		6.8	5.1
		4	3.0	1.3	137		Pre-operative 5/31		2.9	1.6
133	Metastases		9.3	6.2			Pre-operative 6/16		3.0	1.5
		2	14.9	12.4		Post orchiectomy, adrenalectomy and cortisone			5.8	3.4
					Treatment with stilbestrol					
134	Localized		1.5	0.1						
		1	1.3	0.0						
135	Localized		3.1	1.7	138				1.9	0.7
		9	3.6	2.2	139				1.1	0.3
								5	0.5	0.0
								18	0.5	0.2
					140				0.9	0.3
					141	Orchiectomy			1.0	0.5
					142				0.9	0.0
					143				0.5	0.0
					144				1.8	0.1
					145				1.0	0.2

* Total and prostatic acid phosphatase are expressed in King-Armstrong units per 100 ml. serum.
† Days after first phosphatase determination.

RESULTS

Serum "prostatic" acid phosphatase in aged chronically-ill men and women

A study was made of the normal distribution of the prostatic serum acid phosphatase in a group of 100 elderly male and 50 female patients hospitalized in institutions for the chronically ill. Figure 1 shows the distribution curves obtained. The striking features are the absence of any values of more than 0.5 units of "prostatic" acid phosphatase in these patients, and the difference between the distribution of the serum enzyme values in men and in women.

Benign prostatic hypertrophy, chronic prostatitis and non-prostatic cancer

In 123 patients distributed in these categories, only rarely (in 3 individuals) did a value for "prostatic" acid phosphatase greater than 0.5 units per 100 cc. of serum appear (Table I).

Prostatic massage

The effects of prostatic massage with respect to the serum acid phosphatase were variable (Figure 2). However, the "prostatic" fraction showed increases following massage which exceeded the normal levels more often than did the "total" acid phosphatase. Thus, in four of the seven cases cited, the "prostatic" and phosphatase rose above 0.5 units whereas in only two cases did the total acid phosphatase rise above 3.5 units.

Cancer of the prostate

Untreated cancer of the prostate. Twelve patients were studied: In seven, metastatic lesions in bone were demonstrated by x-ray, in four only

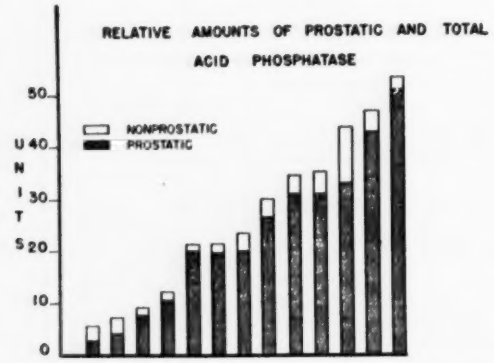

FIG. 3. INDIVIDUAL SERA OF PATIENTS WITH CANCER OF THE PROSTATE, ARRANGED IN ASCENDING VALUES OF TOTAL ACID PHOSPHATASE

Note the large proportion of L-tartrate-inhibited acid phosphatase. Units are the conventional King-Armstrong units.

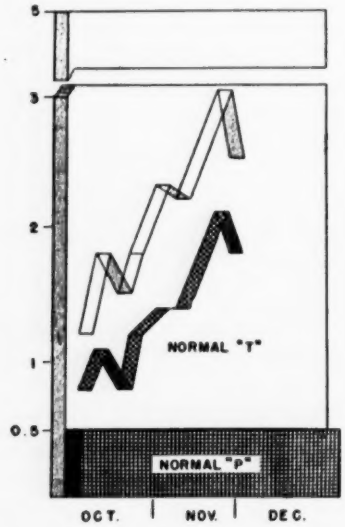

FIG. 4. PATIENT B. TOTAL ACID PHOSPHATASE (T) AND "PROSTATIC" ACID PHOSPHATASE EXPRESSED IN KING-ARMSTRONG UNITS PER 100 CC. SERUM IN A PATIENT WITH CLINICAL CANCER OF THE PROSTATE WITHOUT SKELETAL METASTASES

The light ribbon represents total and the checkered ribbon "prostatic" acid phosphatase. The normal limits for each are shown in the light and dark shaded areas. In every instance the "prostatic" component was in the pathological range, while the total acid phosphatase was well within normal limits.

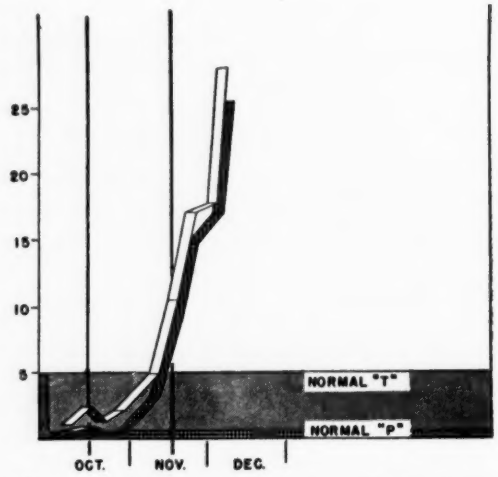

FIG. 5. A STUDY OF A PATIENT WITH CANCER OF THE PROSTATE SHOWING SKELETAL METASTASES ILLUSTRATING THE ELEVATION IN THE "PROSTATIC" ACID PHOSPHATASE OF THE SERUM (CHECKERED RIBBON) WHICH PRECEDED THE RISE IN TOTAL ACID PHOSPHATASE (LIGHT RIBBON) IN KING-ARMSTRONG UNITS

localized prostatic lesions were present, and in one the possibility of metastases could not be ruled out. In this series of patients, both total and "prostatic" acid phosphatase values were found in the pathological range in the seven subjects with metastases (Table II).

On the other hand, five patients with proven prostatic cancer without evidence of metastases (Patients 130, 131, 132, 134, 135) gave values for acid phosphatase between 1.3 and 3.0 King-Armstrong units, *i.e.*, well within the normal range. *Of these five subjects, four gave results for the serum "prostatic" acid phosphatase of 0.7 to 2.2 units.* These are regarded at this time as abnormal values. The fifth patient gave results for both types of serum enzyme within normal limits (Patient 134). The prostatic pathologic lesion was as follows:

Patient No.

(Co) 130: Rectal examination showed a hard nodular prostate markedly enlarged and fixed to the pelvic wall. Needle biopsy: adenocarcinoma.

(Pa) 131: Histologic examination after radical prostatectomy, in spite of negative needle biopsy, showed adenocarcinoma of the prostate. Gross description: prostate gland together with prostatic urethra and seminal vesicles

showing carcinoma involving the left lobe of the prostate and base of the left seminal vesicle.

(Bu) 132: Transurethral prostatectomy three years ago (Massachusetts General Hospital) showed adenocarcinoma of the prostate. The prostate is at present rocky hard and four to five times normal size. Clinically, cancer of prostate.

(Do) 134: The pathological specimen showed a localized carcinoma of 1 cm. in diameter, infiltrating the prostatic capsule and the wall of one seminal vesicle.

(Wa) 135: Clear-cut localized cancer of the prostate by clinical examination.

Figure 3 illustrates the fact that in all instances where the total serum acid phosphatase was found elevated, this increase of enzymatic activity was due largely to the "prostatic" component.

Estrogen therapy in prostatic cancer (*Table II*). All eight patients with proven carcinoma of the prostate whose disease was controlled by estrogen therapy showed normal values for the total acid phosphatase ranging from 0.5 to 1.9 units. One of these had an abnormal "prostatic" value (0.7).

Surgery and prostatic cancer. Four patients were followed after surgical operations (Table II). The pre-operative studies on two of these have already been discussed (Patients 130, 131).

In Patient 130, who underwent bilateral orchiectomy, the serum acid phosphatase was never abnormal. However, the "prostatic" fraction was elevated (0.7 to 1.3 units) before the orchiectomy and fell thereafter, reaching a normal level (0.2 units) some three months after this operation.

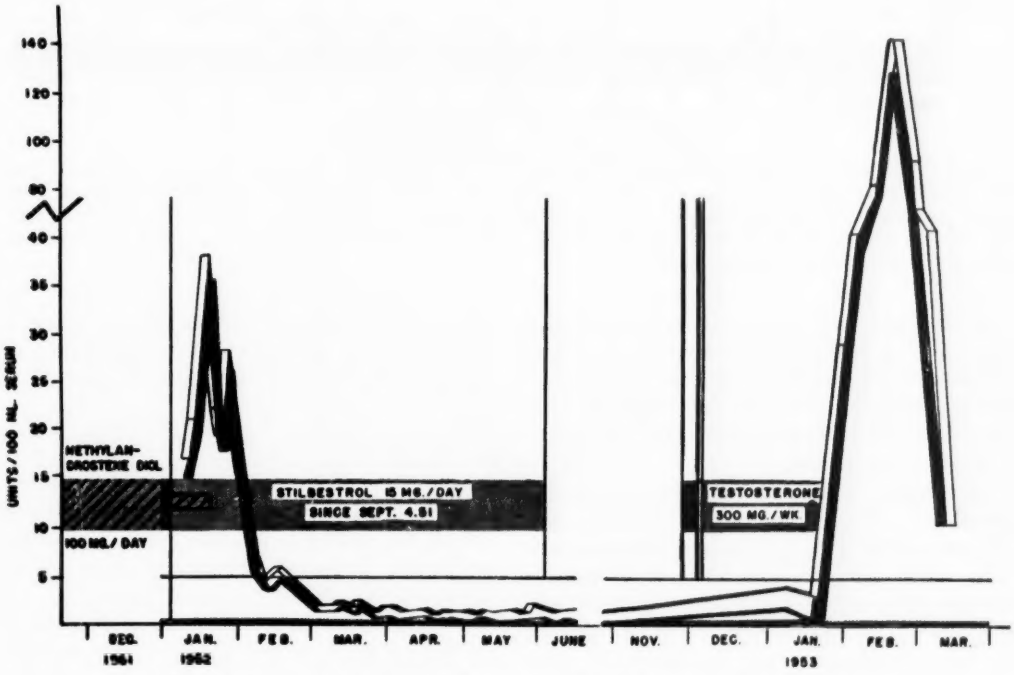

FIG. 6. CLINICAL SUMMARY. PATIENT C., WHITE, MALE, 75 YEARS OLD

Urinary obstruction and clinical evidence of cancer of the prostate in 1950. Osteoblastic and osteolytic metastases to pelvis and spine. First estrogen therapy was given in 1950, later interrupted, and resumed September 4, 1951. Between November, 1950, and April, 1951, there was marked progression of metastases in spite of x-ray therapy. Orchiectomy was refused. The dosage of Stilbestrol used varied from 10 to 15 mg. per day and was well tolerated. The patient, who also suffers from generalized arteriosclerosis, is at present quite well clinically without symptoms referable to his prostatic tumor (enlarged, hard, nodular prostate) and in spite of x-ray evidence of widespread metastases. Light ribbon is T phosphatase and checkered ribbon is P phosphatase. Note the changes of acid phosphatase occurring with various androgen therapy.

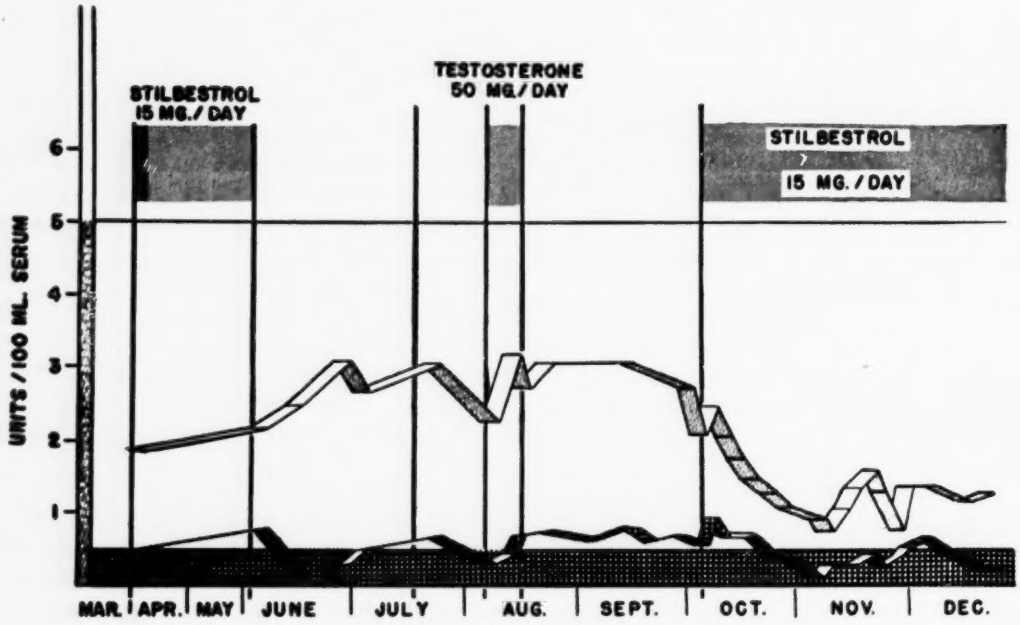

FIG. 7. PATIENT N., WHITE, MALE OF 80

Cancer of the prostate diagnosed January, 1951, by rectal examination. At that time there was evidence of anemia, hgb., 9 Gm., and elevated serum acid phosphatase, but x-ray studies did not reveal metastases. Repeated x-ray studies one month later showed osteolytic and osteoblastic metastases in right femur. May, 1951, fracture of right hip. Course from beginning of study (March, 1953) has been progressively downhill. Light ribbon is T phosphatase and checkered ribbon is P phosphatase.

In the presence of a normal acid phosphatase value in Patient 131 the "prostatic" component was elevated before a radical prostatectomy and bilateral vasoligation was done and showed a trend towards normal levels thereafter, which may, however, not be significant.

In Patient 136 both "prostatic" and total acid phosphatase remained in the pathological range following orchiectomy and bilateral adrenalectomy.

In Patient 137 (previous bilateral orchiectomy and now bilateral adrenalectomy) the serum acid phosphatase was not abnormal until some time after the last operation, whereas the "prostatic" component was elevated (1.6 to 3.4 units) throughout the course of the disease.

In several patients who were studied following total prostatectomy, "prostatic" acid phosphatase levels of up to 0.5 units have been observed although lower values than this were the rule. This amount is believed to represent non-prostatic sources of acid phosphatase which are inhibited by L-tartrate (11). In our experience so far, this non-prostatic source does not interfere in the interpretation of the values for "prostatic" acid phosphatase.

Long-term study of cancer of prostate

Long-term studies comparing results of conventional serum acid phosphatase determinations and the present method in prostatic cancer in patients undergoing hormonal therapy are illustrated in Figures 4 through 9.

The clinical histories in summary of the patients studied are found in the legends of these figures.

No therapy. Six patients have been studied. Two typical untreated cases are illustrated in Figures 4 and 5. The two enzyme levels were found to parallel each other. However, in Figure 4 it is evident that throughout a three-month period the serum total acid phosphatase was within the normal range, even though rising, whereas the "prostatic" component was in the pathological

range from the very beginning of the observation period (see also Table III). Figure 5 shows that in another patient the level of "prostatic" acid phosphatase indicated an abnormal value more than a month before there was any abnormality evident from study of the serum total acid phosphatase.

Hormonal therapy. Figures 6, 7, and 8 illustrate the point that the "prostatic" acid phosphatase level reflects in identical fashion to the serum total acid phosphatase the response or sometimes lack of response of the enzyme to hormone action. In addition, it seems clear that the "prostatic" component provides a more sensitive and accurate index of activity of prostatic cancer. Figure 8 shows a marked spontaneous downward change towards normal levels which occurred simultaneously in both enzyme fractions. Stilbestrol, which was given later, did not further depress the serum enzyme activity. The failure of stilbestrol to do so is also illustrated in Figure 9, showing parallel

TABLE III

*Patient B. A systematic long-term study of total and "prostatic" acid phosphatases in a patient with clinical cancer of the prostate but without evidence of skeletal metastases**

Date	Serum acid phosphatase Total	"Prostatic"	Date	Total	"Prostatic"
1952			1953		
10/9	1.2	0.8	1/9	2.4	1.4
10/16	1.8	1.1	1/19	3.3	2.3
10/23	1.5	0.8	1/30	3.2	2.3
10/28	1.8	1.2	2/5	2.3	1.6
11/6	2.3	1.4	2/12	2.9	1.7
11/13	2.2	1.4	2/18	3.4	2.4
11/28	3.1	2.1	2/27	3.6	2.5
12/4	2.5	1.8	3/5	3.0	2.2
12/15	2.2	1.6	3/13	2.3	1.5
12/18	3.0	2.3	3/20	2.4	2.0
12/31	2.0	1.0	3/27	2.9	2.2

* The observations supplement those illustrated in Figure 4.

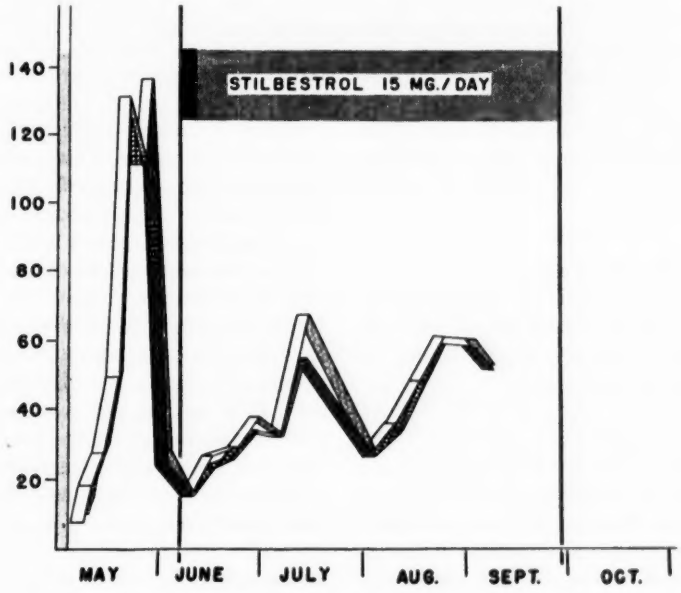

FIG. 8. PATIENT DI P., WHITE, MALE OF 72

In good health until March, 1952, when gross hematuria was noted. There was an enlarged, stony-hard prostate and elevated acid phosphatase. The patient was transferred to the Holy Ghost Hospital from another institution where no therapy had been given. No hematuria was found on admission, and the examination of the prostate was as noted. X-ray series at time of admission showed osteolytic lesion in the seventh dorsal vertebra and in the left ramus of the pubis. The patient was ambulatory throughout his hospitalization. Light ribbon is T phosphatase and checkered ribbon is P phosphatase in King-Armstrong units.

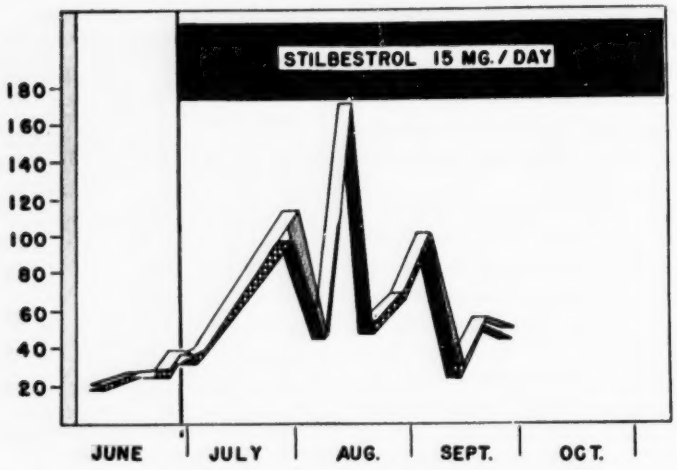

FIG. 9. PATIENT H., COLORED, MALE OF 87

Generalized arteriosclerosis, pain in pelvic and lumbar areas, enlarged and tender prostate with nodular induration on right upper pole. Elevated acid phosphatase and biochemical course shown in Figure 9. Clinically there was marked improvement during Stilbestrol therapy with disappearance of pelvic and lumbar pain and return of patient to normal activity. Light ribbon is T phosphatase and checkered ribbon is P phosphatase in King-Armstrong units.

fluctuations at highly pathological levels in a patient during clinically unsuccessful estrogen therapy.

DISCUSSION

The levels in the blood serum of acid phosphatase elaborated by normal and neoplastic prostatic cells are subject to a great many regulating factors. To name a few, one must consider the rate of enzyme production by the cells, the permeability of the cells, the solubility of the enzyme, the circulatory and anatomical conditions in and around the cancerous prostate and its metastases, possible activating or inhibiting substances in tissues, lymph, and blood stream, the rate of diffusion from the vascular channels and the rate of absorption of the enzyme by various tissues, its destruction by such tissues and perhaps its excretion from the body. In spite of the complexity of this physiological situation, determinations of serum acid phosphatase by the King-Armstrong method have become of considerable usefulness to the clinician dealing with prostatic cancer. The clinical specificity of this determination is considerable but as was pointed out in the introduction, its sensitivity is of low degree.

Serum "prostatic" acid phosphatase. The evidence that, by means of the L-tartrate inhibitor as employed in the present study, one measures largely but not exclusively, prostatic acid phosphatase, is based on both experimental and clinical data.

Complete inhibition of prostatic gland acid phosphatase is obtained *in vitro* with L-tartrate (8, 9). In addition, this inhibitor is fully active on prostatic acid phosphatase added to human sera (8, 9).

It appears that in man the blood serum contains very little acid phosphatase from non-prostatic sources which is inhibited by L-tartrate. Thus, the sera of women contains very often not a trace of L-tartrate-inhibited acid phosphatase. The small amounts which are present in other women are undoubtedly due to enzyme from organs such as spleen or liver (9) which is inhibited by L-tartrate.

Clinical evidence supports the view that the prostate gland is the predominant source in the serum of L-tartrate-inhibited acid phosphatase. Thus, prostatic massage results in a significant increase of the fraction of serum acid phosphatase inhibited by L-tartrate more often than of the total acid phosphatase. The fact that palpation of the prostate in 10 per cent of patients without cancer of the prostate does give rise to a subsequent increase in

serum acid phosphatase is known (10, 11). In cancer of the prostate the "prostatic" acid phosphatase in the serum is elevated on every occasion when one encounters a high serum acid phosphatase. Serum "prostatic" acid phosphatase behaves in a parallel fashion to the total acid phosphatase under varying regimes of sex hormone therapy.

Clinical sensitivity of "prostatic" acid phosphatase in serum. The serum level of this "prostatic" acid phosphatase appears to be a far more sensitive index of prostatic activity than is the serum level of the total acid phosphatase determined by the King-Armstrong method.

This is deduced from the observations that the effects of prostatic massage are not evident from the total enzyme levels as often as from the "prostatic" serum enzyme levels, that the "prostatic" serum acid phosphatase was found increased in some cases of proven cancer of the prostate without demonstrable bone involvement at a time when there were completely normal levels of the total serum acid phosphatase, and from the fact that the "prostatic" serum enzyme rose with androgen administration and fell with estrogen therapy at times when no significant changes occurred in the total serum acid phosphatase.

The readiness with which the L-tartrate inhibited enzyme enters the blood stream from the prostate after palpation, a fact which renders this procedure more sensitive than the conventional method, also calls for great caution in avoiding the taking of blood specimens for this determination within several hours after the prostate has been manipulated.

The observations reported in this paper are presented with the conclusion that this method probably comes closer to measuring true prostatic acid phosphatase in serum than do previous ones.

SUMMARY

1. The principle of inhibition of an acid phosphatase fraction in prostatic tissue by L-tartrate has been applied to the serum acid phosphatase in patients with various non-prostatic diseases and in patients with benign prostatic hypertrophy, chronic prostatitis and proven cancer of the prostate.

2. The normal distribution of this serum enzyme fraction in elderly men and women has been determined, 0 to 0.5 units per 100 ml. serum.

3. Palpation of the prostate was followed by a transient rise in "prostatic" acid phosphatase of serum (up to 1.5 units per 100 ml. serum) in the absence, as a rule, of abnormal values of serum total acid phosphatase.

4. In untreated cancer of the prostate, seven patients with bone metastases exhibited abnormal values for both total and "prostatic" serum acid phosphatase.

5. In four out of five patients with untreated cancer of the prostate without demonstrable metastases, abnormal values for "prostatic" acid phosphatase in the presence of normal serum acid phosphatase levels were observed.

6. Long-term studies of hormonal therapy of cancer of the prostate gland demonstrate a close parallel behavior of both total and "prostatic" serum acid phosphatase.

7. In these studies, abnormal "prostatic" acid phosphatase values in serum preceded and succeeded the appearance and subsequent disappearance of pathological levels of the total acid phosphatase.

8. On the basis of the present data, it is considered that the clinical application of the method of Fishman and Lerner for "prostatic" acid phosphatase in serum may offer real advantages over previous ones.

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OBSERVATIONS ON PAIN PRODUCED BY INJECTION OF HYPERTONIC SALINE INTO MUSCLES AND OTHER SUPPORTIVE TISSUES

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To study the clinical effects of experimentally induced pain, as initiated by Kellgren and Lewis, hypertonic saline was injected into selected areas of muscle and about certain tendons and ligaments in a number of individuals (1–3). The resulting subjective sensations and the nature and extent of the objective changes were noted, then tabulated and compared by coworkers not participating in the experiments. This report presents our observations and deductions.

METHOD

Acute pain was produced by the injection of hypertonic saline solution into various selected anatomic sites. These consisted of the scalenus anticus, supraspinatus, trapezius, deltoid, gluteus medius, and erector spinae muscles; the achilles, sacroiliac interspinous, and insertional biceps tendons, and areas about the knee joint.

A 6 per cent solution of sodium chloride in distilled water was used, as in the investigations of Kellgren (2). The amount of hypertonic saline solution injected at each point varied from 0.2 to 0.5 cc.

Eighteen individuals served as subjects for a total of 61 trials. Most of the volunteers submitted to several determinations. The number of trials for each participant is summarized in Table I.

TABLE I

Number of trials per individual

Number of trials per individual subject	10	5	4	3	2	1
Number of subjects	2	1	3	3	6	3

This study group consisted of healthy adults who were either physicians, medical students, or nurses. The common medical background reduced to a great extent any emotional factor incident to the use of the needle and syringe.

Subjects were not briefed as to what to expect before the actual testing. Each was required to write immediately a detailed description of such local and referred sensations as occurred. Procaine anesthesia as a preliminary to the infiltration of saline was omitted in order to avoid artificial effects at the point of entry and adjacent areas. Most subjects disregarded the initial needle prick.

Two observers checked each individual for objective sensory and other changes before and after infiltration. Superficial hyperesthesia was determined by pinch, prick, and scratch, and a pressure gage was used to elicit deep tenderness. Comparisons were made each time with symmetrical areas on the opposite side of the body. Alterations in skin temperature were determined by use of the Tycos thermocouple. Careful observations also were made for variations in skin color, sweating, and visible or palpable muscle contraction. The results have been recorded in Table II.

OBSERVATIONS

The musculosensory responses to the stimuli used by us may be divided into two categories: the *primary reaction* and certain associated phenomena to be enumerated and discussed later. The *primary reaction*, the immediate pain at the point of injection, was always present.

The quality of the primary reaction or "local pain" was found to be quite diversified, with no characteristic sensation for any one type of tissue or site of stimulation. The pain at the site of injection was variously described as "sharp," "dull," "severe aching," "throbbing," "stinging," or "burning." This applied to muscle (trapezius), tendon (biceps), and ligament (sacroiliac), as well as to a "complex region" such as the knee joint. It was found, moreover, that even in the same individual the quality of local pain subsequently might change from trial to trial.

The intensity of this local pain was likewise variable, as was its duration, when the same quantity of saline was introduced. Pain or aching at the injection-site lasted from minutes to days, and even recurred in some instances. Cessation usually was gradual, but in some trials at the knee it was strikingly abrupt.

TABLE II
Results of hypertonic saline injections

	Assoc. phenomena (subjective)					Assoc. phenomena (objective)					
Sites of injection	No. of trials	Radiating pain	Paresthesias	"Weakness" of part	Euphoria	Cutaneous sensory change	Deep tenderness	Surface color changes	Skin temperature	Sweating of part	Musc. contraction
Biceps tendon (at elbow)	10	8 (2 near, 6 distant)		2 (at wrist)		1	6 (locally 1, distant 5)	2 (pallor 1, erythem. 1)			
Erector spinae musc.	1	1									
Interspinous ligament	2	2					2 (at site)				
Sacro-iliac ligaments	3	1 (up and down) to knee				1 (gluteal)			2 (decrease of homolat. calf)		
Knee joint	7	6 (up femur and around patella)			4	5 (hypoesthesia 3, hyperesthesia 2)	7 (no radiation)		2 (1 decrease, 1 increase)		
Achilles tendon	6	3 (variable distribution)	1				3 (1 at site, 2 larger area)	3 (pallor)	5 (decrease)	1 (heel)	
Scalenus anticus musc.	7	5 (uni- or bi-directional) shoulder 3 cases, 4th and 5th fingers 1, cerv. 2				6 (4 locally)	4 (at site 3, distant 1)				2 (in trapezius musc.)
Supraspinatus musc.	5	5 (shoulder 3, elbow 1, upper back 2)					3 (at site 2, distant 1)		2 (decreased over trap.)		
Trapezius musc.	14	13 cephalad				3 (2 locally, 1 distant)	8 (at site 6, distant 2)		5 (decreased)		9
Trap. and supraspin.	1 (control)	3			2	1 (at site)	3 (at site)				
Trap. and infraspin.	1										
Deltoid musc.	2	2			2		1				
Gluteus medius	2	1									

The *associated phenomena* consisted of a number of subjective and objective reactions accompanying or following the "primary" pain (Figures 1–3). They did not appear in all cases. They were either local (at or over the site of infiltration) or distant, or both, and appeared either as isolated effects or in unpredictable combinations. The reaction patterns were, therefore, rich or meager, and often rather bizarre. Such associated phenomena included both subjective sensations and objective physiologic changes.

Among the subjective reactions were the following: radiating pain of variable quality, intensity and duration even in the same individual given a similar restimulation, paresthesias, a sense of "weakness" of the extremity, and euphoria.

Radiating pain was usually of the same quality as the local pain. Instances wherein these differed were noted at the trapezius and biceps regions. Like the local sensation, the duration of radiation was variable but did not necessarily correspond to it. The direction or pathway of radiating pain was

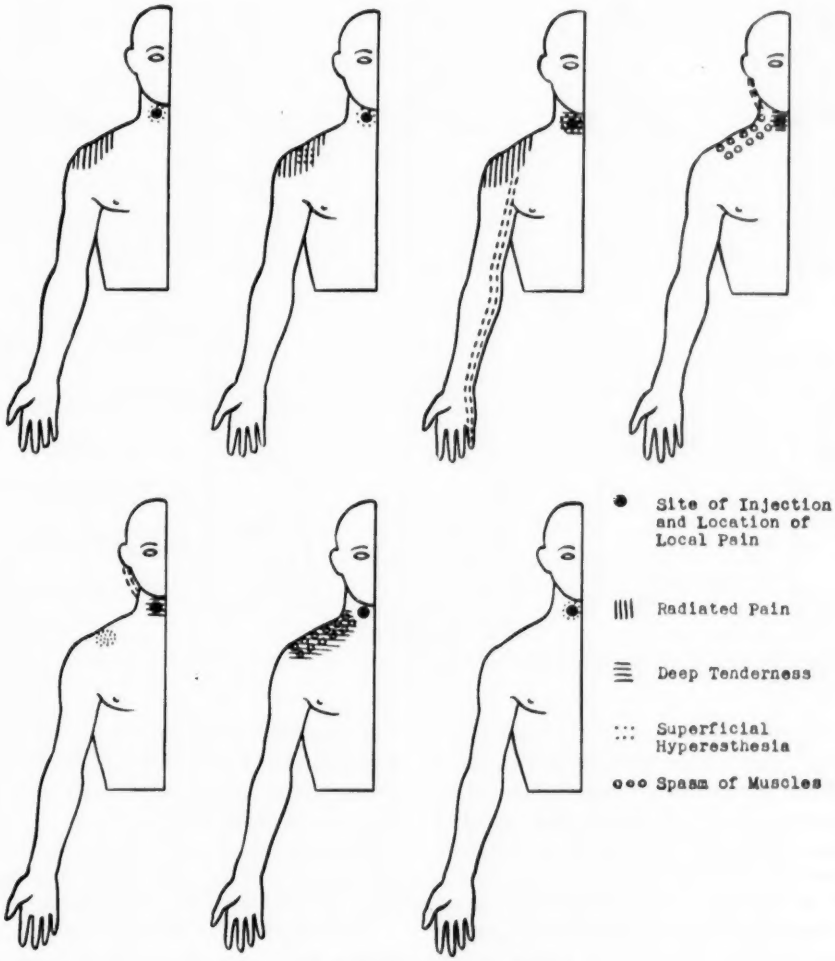

FIG. 1. SCALENUS ANTICUS MUSCLE

Pattern of pain and associated phenomena following injection of the Scalenus Anticus muscle with 6 per cent Hypertonic Saline in 7 subjects.

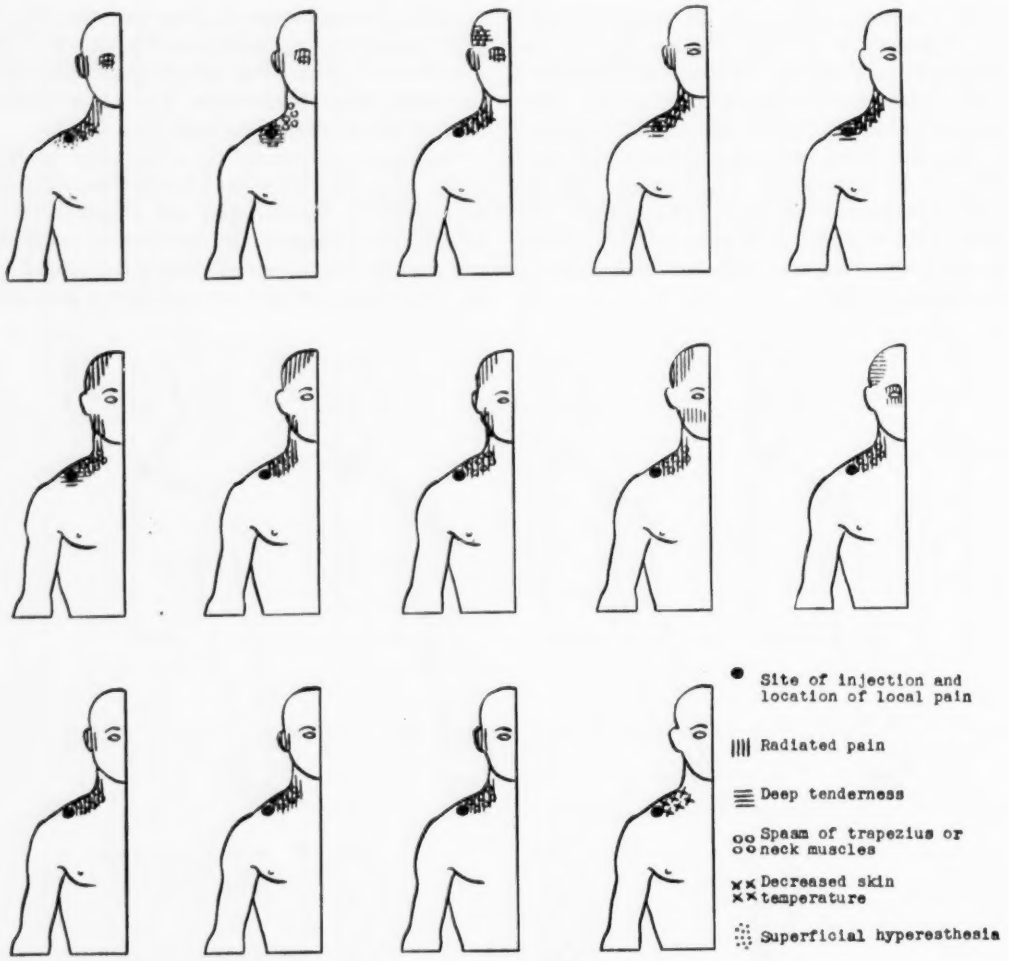

FIG. 2. TRAPEZIUS MUSCLE

Patterns of pain and associated phenomena following injection of Trapezius muscle with 6 per cent Hypertonic Saline in 14 subjects.

peripheral (descending), central (ascending), or bidirectional. Such pathways often were unrelated to any specific or recognizable neural distribution.

Paresthesias, when associated with radiating pain, frequently affected quite different locations.

In some trials they also included sensations of "warmth" or "burning" which occurred in the absence of objective changes of skin temperature, or even when the area of reference was in reality measurably cooler. This was noted especially after injections of the trapezius and periarticular tissues of the knee.

A subjective "weakness" of the distal part of the upper extremity followed injection of the biceps tendon. This symptom occurred both with and without associated radiating pain.

Euphoria, an unusual "feeling of well-being," was experienced by a few subjects as the induced pain subsided, apparently by contrast.

The local objective changes included superficial hyper- and hypoesthesia, deep tenderness, increased and decreased skin temperature, color changes of flushing and pallor, increased sweating and muscular spasm.

Changes in superficial sensation and deep tenderness were inconstant in occurrence. The former usually consisted of hyperesthesia, rarely hypoesthesia. The significance of the fact that hypoesthesia was noted only following injection about the knee joint is not clear. The sites of superficial hyperesthesia and deep tenderness involved either the local area (injection site) or distant regions. Both appeared alone or in association with each other. In some instances the area of deep tenderness corresponded to the course of the radiating pain; in other cases it was unrelated.

There was no correlated occurrence of sensory alterations and temperature changes. The latter

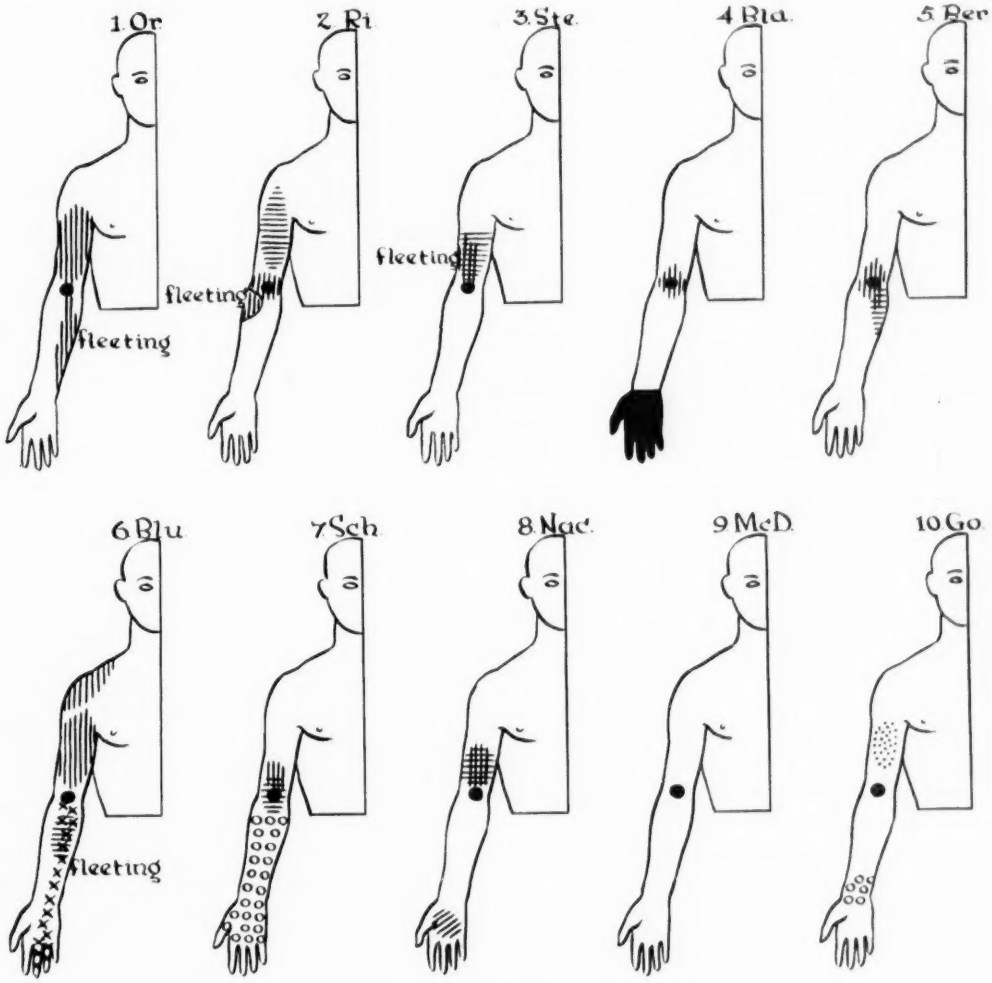

FIG. 3. BICEPS TENDON

- Site of injection and location of local pain
- Radiated pain
- Deep tenderness
- Erythema
- Weakness (subjective)
- Superficial hyperesthesia
- Paresthesia
- Pallor

Patterns and distribution of associated phenomena following injection of the biceps tendon with 6 per cent Hypertonic Saline solution in 10 subjects.

also were variable. In the same region (knee) in different subjects the local skin temperature was both increased and decreased. In general, however, the latter reaction predominated.

Color changes, likewise, were diverse. Both pallor and flushing occurred at a distance in different individuals, for example following injection at the biceps tendon. In other instances pallor was the usual reaction. Changes in surface temperature and color did not necessarily appear together.

Sweating, like color change, was observed at a distance from the injection site.

It is of interest that injections were followed by visible or palpable muscular contraction only in the neck and shoulder regions.

DISCUSSION

In our hands the reactions of supportive tissues to the infiltration of hypertonic saline solution obviously were varied and unpredictable in quality and quantity for each site. In fact in some instances the effects were different even when the procedure was repeated later at a chosen location in the same individual. Our observations show that in some subjects the injection of hypertonic saline into certain supportive structures produces local pain without any demonstrable coincidental effects. In others, however, any of a variety of associated reactions may appear. The pain and associated phenomena suggested a definite segmental or a specific neural pattern in only a few of our trials, in contradistinction to the observations of Kellgren and Lewis. In most of our trials, in fact, the reactions were too complex or too irregular for the identification of typical pathways. Perhaps a larger number of injections of spinal ligaments would provide more data conforming to the patterned segmental distributions deduced by Kellgren and Lewis.

The remarkable diversity of responses elicited by the same stimulus at corresponding sites naturally has raised questions in our minds as to the sources of these variations. To interpret this medley of reaction-patterns it is possible that, in addition to a small number of trials suggestive of segmental distribution, some represent an axone reflex, others the direct stimulation of a peripheral nerve filament or possibly of a sympathetic element. Finally, in a small number there appears to be stimulation of higher or lower segments without evidence from the direct level of irritation. The nature, severity and distribution of response to the stimulus, therefore, would seem to depend on its greater or lesser proximity to any or several of the multiple constituents of the infiltrated tissue—nerve element(s) (spinal or sympathetic—trunk, fibril, or end-organ); blood vessel(s) (trunk or branch, sheath or other layer); fascia, muscular bundles, ligament, subcutis, or periosteum. Whether or not these considerations account for the variability of our observations, the experimental results and the difficulties of interpreting them do not permit some of the generalizations and applications previously derived from similar investigations (2, 3).

A noteworthy observation during these experiments was the absence of any "trigger" mechanisms in the reactive phenomena produced. Palpation at the site of injection or other tender areas, immediately after the infiltration or while discomfort lasted, usually produced local soreness. In no instance, however, did it quite provoke the related features with the pain initially associated with the infiltration. It seems likely that the nature of the stimulus and the effects it aroused were inadequate or too brief in their action to create the "trigger points" and their clinical patterns ascribed to some muscular, myofascial, and musculoskeletal disorders (5–11).

The injection of hypertonic saline into the various supportive tissues in our series has not produced a distinctive reaction-pattern for each anatomic unit which might thereby be recognized clinically, as Kellgren's studies imply. These observations do not provide a basis for the current trend toward the delineation of symptom-complexes or "syndromes" characteristic of lesions of various supportive structures, particularly muscles. Stimulation with hypertonic saline usually evokes rather temporary effects, sometimes quite explosive in intensity and spread. These suggest to the observer, as the most closely analogous clinical situations, the acute and transient symptoms of traumata, or of a moderately potent insect bite. It is quite doubtful whether such acute reactions and their effects may be compared correctly with chronic disorders in which the acute features have subsided and the persistent disturbance and structural changes create physiologic mechanisms and

clinical patterns largely dependent on the factors of time and prolonged stimulation.

Our observations, therefore, indicate that injections of hypertonic saline may be regarded as an adequate stimulus only for the production of acute and transient pain and related phenomena. Furthermore, these responses in our series have neither provided a factual basis for deducing a specific segmental or neural pathway for each anatomic entity stimulated, nor have they constituted distinctive, consistent reaction patterns by which involvement of each structure might be identified in clinical diagnosis.

SUMMARY AND CONCLUSIONS

1. The clinical effects of pain experimentally induced by the injection of hypertonic (6 per cent) solution of sodium chloride, as initiated by Kellgren and Lewis, were studied in 18 volunteers subjected to 61 trials.

2. The material was injected into muscles—the scalenus anticus, supraspinatus, trapezius, deltoid, gluteus medius, and the erector spinae; into or about tendons—the achilles and insertional biceps; at ligaments—the sacroiliac and interspinous and about the knee joint.

3. Injection of hypertonic saline into muscle, about a tendon, or at a ligament caused pain and aching at the point of infiltration, usually associated with one or more of the following phenomena; radiating soreness or shooting pain at a distance, paresthesias, hyperesthesia of overlying skin, and/or at a farther area, greatest tenderness to palpation at the point of injection, lesser soreness to palpation at a distant region, muscle "spasm," stiffness, and increased or decreased cutaneous temperature.

4. These complex but variable reactions were immediately or quickly provoked. Aching at the point of injection lasted for minutes to days. The other features disappeared rapidly when present.

5. Our data may be said to confirm the findings of Kellgren and Lewis only to the extent that pain always, and a local point of greatest soreness often, were induced at the site of injection. These were accompanied only at times by an area of lesser tenderness of skin or muscle about it or at an adjacent or distant location.

6. In some cases the demarcations of pain and tenderness suggested a segmental distribution. In most instances these features were irregular and without the definite segmental or recognizably uniform characteristics described by the original observers.

7. Consistent similar effects from stimulation of each of the various selected sites were too infrequent to deduce predictable clinical reaction patterns typical of individual anatomic structures.

8. Our observations on the acute effects of injecting hypertonic saline failed to provide an experimental basis for such clinical concepts in musculoskeletal pain as "trigger point" mechanisms or specific, diagnostic reaction patterns to identify involvement of individual muscular and other supportive structures.

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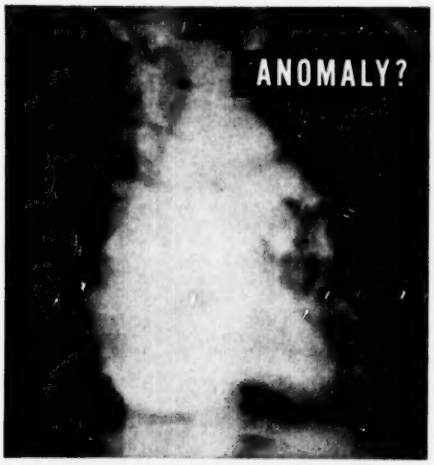

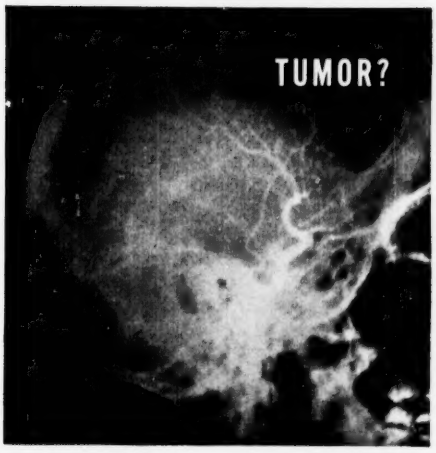

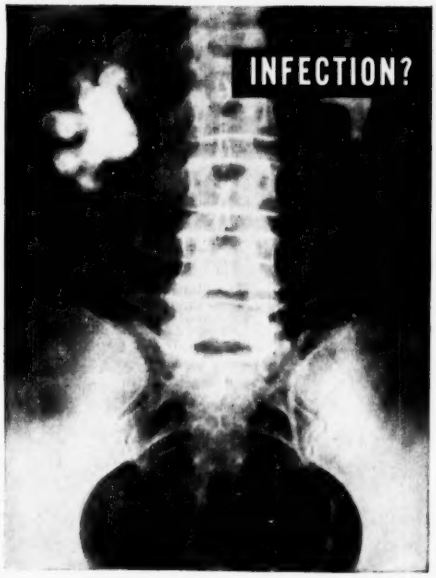

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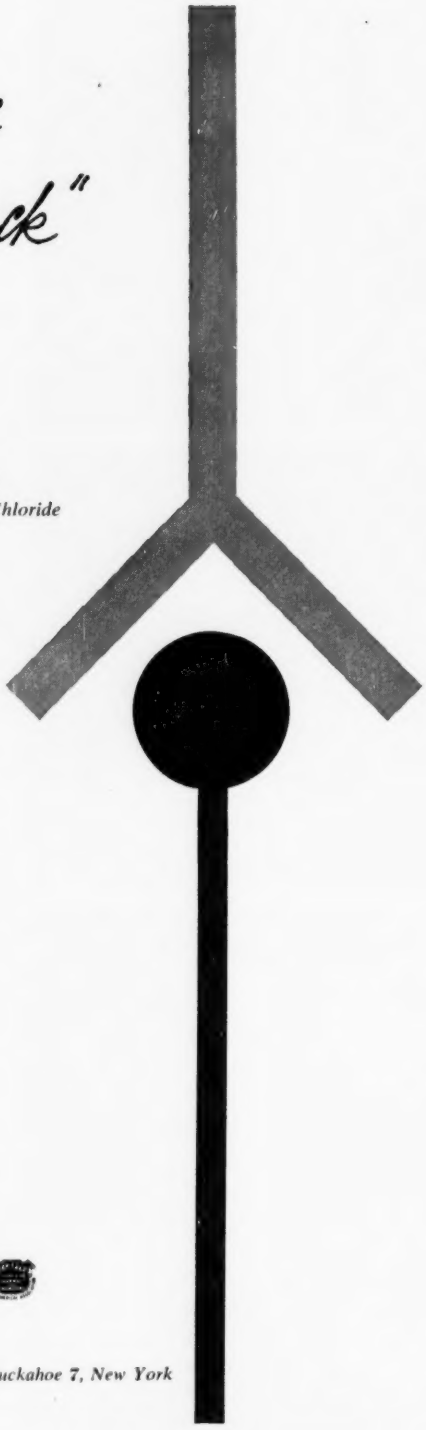

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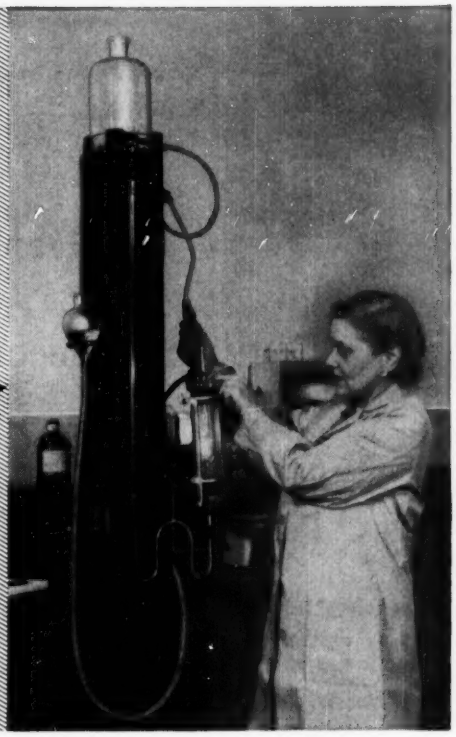

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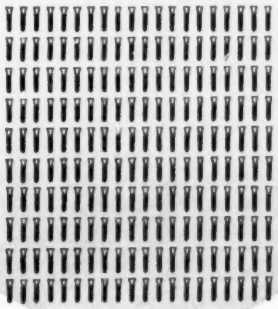

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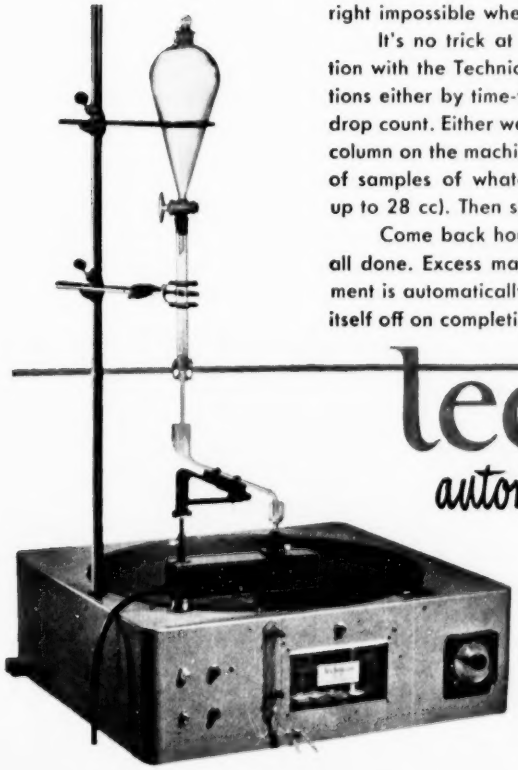

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Each cc. contains 100 mg. of pure crystalline Terramycin. Supplied in 10 cc. bottles with special dropper calibrated at 25 mg. and 50 mg. May be administered directly or mixed with nonacidulated foods and liquids. Economical 1.0 gram size often provides the *total dose* required for treatment of infections of average severity in infants.

Supplied: Bottles of 1.0 Gm.

Oral Suspension *(Flavored)*

Each 5 cc. teaspoonful contains 250 mg. of pure crystalline Terramycin. Effective against gram-positive and gram-negative bacteria, including the important coli-aerogenes group, rickettsiae, certain large viruses and protozoa.

Supplied: Bottles of 1.5 Gm.

Pfizer **PFIZER LABORATORIES,** *Brooklyn 6, N. Y., Division, Chas. Pfizer & Co., Inc.*

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